

THE ROLES OF PANCREATIC HORMONES IN REGULATING PANCREAS  
DEVELOPMENT AND BETA CELL REGENERATION

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THE ROLES OF PANCREATIC HORMONES IN REGULATING PANCREAS  
DEVELOPMENT AND BETA CELL REGENERATION

Diabetes mellitus is a group of related metabolic diseases that share a common pathological mechanism: insufficient insulin signaling. Insulin is a hormone secreted from pancreatic  $\beta$  cells that promotes energy storage and consequently lowers blood glucose. In contrast, the hormone glucagon, released by pancreatic  $\alpha$  cells, plays a critical complementary role in metabolic homeostasis by releasing energy stores and increasing blood glucose. Restoration of  $\beta$  cell mass in diabetic patients via  $\beta$  cell regeneration is a conceptually proven approach to finally curing diabetes. Moreover, *in situ* regeneration of  $\beta$  cells from endogenous sources would circumvent many of the obstacles encountered by surgical restoration of  $\beta$  cell mass via islet transplantation. Regeneration may occur both by  $\beta$  cell self-duplication and by neogenesis from non- $\beta$  cell sources. Although the mechanisms regulating the  $\beta$  cell replication pathway have been highly investigated, the signals that regulate  $\beta$  cell neogenesis are relatively unknown. In this dissertation, I have used zebrafish as a genetic model system to investigate the process of  $\beta$  cell neogenesis following insulin signaling depletion by various modes. Specifically, I have found that after their ablation,  $\beta$  cells primarily regenerate from two discrete cellular sources: differentiation from uncommitted pancreatic progenitors and transdifferentiation from  $\alpha$  cells. Importantly, I have found that insulin and glucagon play crucial roles in controlling  $\beta$  cell regeneration from both sources. As with metabolic regulation, insulin and glucagon play counter-balancing roles in directing endocrine cell fate specification. These studies have revealed that glucagon signaling promotes  $\beta$  cell formation by increasing differentiation of pancreas progenitors and by destabilizing  $\alpha$  cell identity to promote  $\alpha$  to  $\beta$  cell transdifferentiation. In contrast, insulin signaling maintains pancreatic progenitors in an undifferentiated state and stabilizes  $\alpha$  cell identity. Finally, I have shown that insulin also regulates pancreatic exocrine cell development. Insufficient insulin signaling destabilized acinar cell fate and impairs exocrine pancreas development. By understanding the roles of pancreatic hormones during pancreas development and

regeneration can provide new therapeutic targets for in vivo  $\beta$  cell regeneration to remediate the devastating consequences of diabetes.

Ryan M. Anderson, Ph.D., Chair

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## LIST OF ABBREVIATIONS

Arx .....	Aristaless related homeobox
bHLH .....	basic Helix-Loop-Helix
BMP .....	Bone Morphogenetic Protein
Capa II.....	carbonic anhydrase II
DBC .....	Dorsal Bud Cells
DE .....	Definitive Endoderm
dpf .....	days post fertilization
dpa.....	days post ablation
DTA .....	Diphtheria toxin A
EP .....	Endocrine Progenitor
ESC .....	Embryonic Stem Cells
Fgf .....	Fibroblast Growth Factor
Gcgr.....	Glucagon receptor
Gcgra.....	Glucagon receptor a
Gcgrb.....	Glucagon receptor b
GH.....	Growth Hormone
GLP-1 .....	Glucagon-like peptide 1
GLP-1r .....	Glucagon-like peptide 1 receptor
GSIS .....	Glucose Stimulated Insulin Secretion
hpf .....	hours post fertilization
Insra.....	Insulin receptor-a
Insrb .....	Insulin receptor-b
IR.....	Insulin Receptor
IRS1 .....	Insulin receptor substrates 1
IRS2 .....	Insulin receptor substrates 2
Lamb1 .....	LaminB1
LRC.....	Label Retaining Cells
MAPK.....	Mitogen Activated Protein Kinase

MO .....	Morpholino
MTZ .....	Metrodinazole
Neurod1.....	Neurogenic differentiation factor1
Ngn3.....	Neurongenin3
NTR.....	Nitroreductase
Pax4.....	Paired Box 4
PC1.....	Proprotein Convertase1
PC2 .....	Proprotein Convertase1
PDL .....	Partial Duct Ligation
Pdx1 .....	Pancreas and duodenal homeobox 1
PE.....	Pancreatic Endoderm
Ptf1a .....	Pancreas transcription factor 1a
PX .....	Pancreatectomy
ROS.....	Reactive Oxygen Species
STZ .....	Streptozotocin
T1DM.....	Type 1 Diabetes Mellitus
T2DM.....	Type 2 Diabetes Mellitus
VBC .....	Ventral Bud Cells
VEGF-A.....	Vascular Endothelial Growth Factor-A

## **CHAPTER ONE: Introduction**

### **I.A. Diabetes Mellitus and $\beta$ Cell Regeneration**

Diabetes Mellitus is a group of metabolic diseases characterized by hyperglycemia that results from insufficient insulin secretion, insulin action, or both. Due to different mechanisms of pathology, diabetes can be mainly classified into two types: Type 1 diabetes Mellitus (T1DM) and Type 2 diabetes Mellitus (T2DM) (3). The development of T1DM is mainly due to the destruction of  $\beta$  cells by autoimmune attack which leads to absolute insulin deficiency. Severe hyperglycemia and appearance of auto-immunity antibodies are usually found in T1DM patients (4). In contrast, patients with T2DM usually have elevated blood glucose levels, but normal or elevated insulin levels. T2DM is strongly associated with genetic disposition and results from the progressive impairment of  $\beta$  cell function and loss of  $\beta$  cell mass in the setting of insulin resistance (5). In addition to these two main types of diabetes, the prevalence of gestational diabetes mellitus (GDM), the induction of maternal insulin resistance and hyperglycemia during pregnancy, has been increasing over time, and is associated with long term impact on the metabolic profiles of the child (6)

#### **i. Current therapeutic approaches for diabetes treatment**

The pathological differences between T1DM and T2DM require different therapeutic interventions. Currently, insulin or insulin analogue treatments are the main approach for T1DM. The administration of exogenous insulin is essential for glycemic control and survival of people with T1DM (7), and although such therapies have dramatically reduced its mortality, several disadvantages limit this approach. First, the requirement for frequent insulin injection not only decreases quality of life, but also imposes huge long-term financial burdens, both at a personal level, and upon the public health system (8). Secondly, exogenous insulin administration cannot perfectly mimic endogenous  $\beta$  cell function, and chronically high levels of peripheral insulin resulting from insulin injections will exacerbate insulin resistance in the peripheral tissues (9,10). Finally, and

most importantly, exogenous insulin administration does not cure the disease. Whether patients are using manual insulin injections or an insulin pump, hyperglycemia will return upon cessation of insulin therapy (11).

As compared with T1DM, more therapeutic options have been developed for patients with T2DM. For most T2DM patients, therapy starts with lifestyle changes including weight loss and exercise (12). When these changes alone cannot achieve the goal of glycemic control, metformin is usually selected as the first-line treatment due to its efficacy, safety, and low cost (13). Eventually for most T2DM patients, metformin must be combined with other pharmaceutical treatments that target various physiological processes including insulin secretion, peripheral tissue insulin uptake, renal glucose retention, and intestinal glucose absorption (14). Besides these, bariatric by-pass surgery has also been used to improve the metabolic profile and glycemic control of T2DM patients with severe obesity (15). However, in most cases, these treatments can only temporarily decrease hyperglycemia, and are not able to permanently reverse T2DM (16). Due to the progressive nature of T2DM and the decline of  $\beta$  cell function and mass over time, many patients with T2DM eventually require insulin therapy to achieve glycemic control (14). In summary, although current therapeutic strategies have enhanced glycemic control and reduced mortality for patients with diabetes, none of these therapies represents a permanent cure. Exogenous insulin administration is required for both T1DM and T2DM at late stages that present with severe  $\beta$  cell deterioration. Thus, in spite of the etiological differences between T1DM and T2DM, loss of  $\beta$  cell quantity and function underlie the pathology and development of both forms of the disease. **Therefore, understanding how to restore  $\beta$  cell function and increase  $\beta$  cell mass and thereby provide a permanent cure to diabetes through recovery of endogenous insulin secretion has become a primary goal of metabolic research (17).**

## **ii. $\beta$ cell regeneration: a pathway toward curing diabetes**

In theory, restoration of sufficient  $\beta$  cell mass could be achieved by augmentation of  $\beta$  cell number from either endogenous or exogenous sources. The former includes the stimulation of endogenous repair mechanisms, while the latter includes transplantation of

islets, differentiated  $\beta$  cells, or undifferentiated  $\beta$  cell progenitors. The first clinical attempt at islet transplantation in T1DM patients was carried out in the 1980s; this transplantation protocol has significantly advanced in recent years, which has brought hope for the cure of diabetes (18). In the most successful studies, about 60% of patients remained insulin independent for one year after islet transplantation, and that 10% remained insulin independent at 5 years (18). Besides T1DM, increasing evidence has also demonstrated the efficiency of pancreas transplantation in the treatment of T2DM (19). In fact, pancreas transplantation in Type 2 diabetics complicated with end-stage renal disease results in outcomes as successful as those patients with T1DM (20). These encouraging results have bolstered the hypothesis that replenishment of pancreatic  $\beta$  cells reverses the course of diabetes, and is so far the closest approximation to a cure.

However, there are still great challenges with the islet transplantation strategy (21). First, there is a great shortage of donor islets. Second, immunosuppression is required to prevent allogenic rejection-induced islet cell death and to block the triggering of autoimmunity. This prolonged immunosuppression not only increases the risk of infection and cancer, but also dampens endogenous  $\beta$  cell regeneration in mouse models (22), which may actually worsen diabetes progression. In the past decade, researchers have focused on stem cells as a source for generating unlimited  $\beta$  cells *in vitro* to overcome these obstacles, and great progress has been achieved towards this goal. By mimicking events of embryonic pancreas development, human embryonic stem (hES) cells or induced human pluripotent stem cells (hiPS) can be directed to differentiate into definitive endodermal cells (23). These cells can be further differentiated into pancreatic endodermal cells, the progenitors of  $\beta$  cells. When transplanted into mice, these pancreatic endodermal cells efficiently differentiate into functional  $\beta$  cells (24). Moreover the implantation of these hES derived pancreatic endodermal cells can fully reverse diabetes in a mouse model (24). Clinical trials using hES-derived pancreatic endodermal cells and an immuno-protective delivery system are underway to evaluate the efficiency and safety of this approach in humans (25). More recent findings have further pushed the differentiation of pancreatic endodermal cells toward mature  $\beta$  cells *in vitro* (26). These *in vitro* differentiated  $\beta$  cells express markers of endogenous  $\beta$  cells, secrete insulin in a glucose-regulated manner, and critically, transplantation of these cells ameliorated

hyperglycemia in diabetic mice (26). Altogether, these findings have generated great optimism for the future of diabetes therapies that restore  $\beta$  cell mass via generation and transplantation of functional insulin-producing cells.

Despite these advances, significant challenges remain. These include surgical risks, immune suppression, the risk of tumor formation from stem cells, and the ethical hurdles associated with acquiring human embryonic stem cells (27). Moreover, transplantation to the liver through the portal vein is currently the most common site for islet transplantation; however, this location does not provide the same innate pancreatic islet environment necessary to restore accurate regulation of blood glucose (28,29). For all of these reasons, promoting endogenous  $\beta$  cell regeneration in the pancreas will have many advantages over *in vitro* artificial  $\beta$  cell generation and transplantation.

### **iii. Evidence for the existence of endogenous $\beta$ cell regeneration in humans**

Multiple studies have shown that the majority of long-duration T1DM patients have detectable C-peptide levels and endogenous insulin secretion (30-32). Further, the secretion of C-peptide increases after a meal, indicating the presence of functional  $\beta$  cells in these patients (30). These observations imply that a small percentage of  $\beta$  cells escape from autoimmune destruction, and/or that continuous, low-level  $\beta$  cell regeneration occur in these T1DM patients. The latter hypothesis of endogenous  $\beta$  cell regeneration is also supported by indirect evidence that  $\beta$  cells are present in long-duration T1DM patients, in spite of on-going apoptosis (33). However, the frequency of residual C-peptide decreases with time, indicating that the endogenous  $\beta$  cell regeneration may eventually become exhausted (32). In support of the hypothesis that endogenous  $\beta$  cell regeneration occurs early in disease progression, the replication rate of  $\beta$  cells is significantly higher in on-set T1DM patients than in age-matched controls and  $\beta$  cell replication rates decline with the progression of disease (34). However, even though these studies support the hypothesis that endogenous  $\beta$  cell regeneration exists in human T1DM patients, clearly regeneration is very limited as it is incapable of preventing diabetes progression (35).



In T2DM, it is also well recognized that  $\beta$  cells can regenerate and compensate for increased physical demands in early stages following the on-set of insulin resistance (36). However, for those who proceed to develop frank diabetes, this  $\beta$  cell adaption is temporary and  $\beta$  cell mass ultimately degenerates (36,37). While the ability to regenerate persists after the onset of T2DM, continual metabolic or physiologic stresses may inhibit endogenous  $\beta$  cell regeneration (38). This insufficiency of  $\beta$  cell regeneration may therefore be a tipping point for T2DM development.

Therefore, in both T1DM and T2DM patients, the ability to regenerate new  $\beta$  cells exists at early disease stages; however, as this capacity becomes exhausted, the disease progresses. For decades, it has been believed that  $\beta$  cell depletion is due to a mechanism of increased  $\beta$  cell death triggered by intolerable environmental and intracellular stresses. However, recent findings suggest that this may not be the only cause. Studies from two independent groups have shown that when  $\beta$  cells lack critical metabolic sensors, e.g. FoxO transcription factors or the  $K_{ATP}$  channel Kir6.2,  $\beta$  cells undergo dedifferentiation rather than apoptosis (39,40). These  $\beta$  cells degranulate and begin to express a gene expression profile similar to pancreatic progenitors. These observations indicate that  $\beta$  cell fate is not rigidly fixed, and that endocrine cells actively adapt to environmental stress to prevent further cellular metabolic stress (39,40). If this mechanism is translatable to human diabetes, novel therapeutic strategies will be needed to coax these dedifferentiated  $\beta$  cells back to a differentiated state. **Nevertheless, finding mechanisms to augment endogenous  $\beta$  cell regeneration or awaken new cellular sources will provide promising future directions for both T1DM and T2DM treatments.**

### **I.B. Pancreas development: from progenitor to endocrine and exocrine lineages**

It is clear that many aspects of tissue regeneration mirror embryonic development (41-43). Therefore, understanding the cellular and molecular mechanisms of  $\beta$  cell formation during embryonic development is critical for developing new therapies that promote endogenous  $\beta$  cell regeneration in children and adults with diabetes. In vertebrates, insulin-secreting  $\beta$  cells and other hormone secreting endocrine cells, including glucagon-secreting  $\alpha$  cells, somatostatin-secreting  $\delta$  cells, pancreatic

polypeptide-secreting PP cells and ghrelin-secreting  $\epsilon$  cells, cluster together to form islet-like structures scattered within the pancreatic exocrine tissues (44). Among all the pancreatic endocrine cells,  $\beta$  cells and  $\alpha$  cells are the two most plentiful endocrine cell types and together they make up ~95% of islet cell mass (44). The exocrine pancreatic compartment comprises acinar cells and duct epithelium cells, and is responsible for the synthesis and secretion of digestive enzymes into the duodenum (44).

Although the pancreas is a complex organ composed of different cell types that perform different physiology functions, all of these cells originate from one group of progenitors in the foregut endoderm during the early organogenesis. Therefore,  $\beta$  cell formation during development is studied within the scope of whole pancreas differentiation program.

#### **i. The morphological aspects of pancreas development**

The morphology of pancreas development is represented in Figure 1-1. A series of signals released from nearby mesoderm and ectoderm imposes patterning on the foregut endoderm, which gives rise to stomach, duodenum, and accessory gut tissue like liver and pancreas. In mammals, pancreatic progenitors bud out from two discrete areas of the foregut endoderm, and are termed the dorsal and ventral pancreatic buds. The ventral pancreas progenitors arise from the posterior ventral foregut, which also gives rise to the liver and gall bladder, while dorsal progenitors evaginate from the dorsal foregut adjacent to duodenum and stomach progenitors (45,46). Although the signals that initiate these two pancreatic buds are different, the progenitor cells themselves have similar gene expression profiles, and both can differentiate into endocrine and exocrine pancreatic cells (44). In successive developmental stages, the closure and rotation of the endodermal tube, together with the uneven growth of the duodenum wall, leads to fusion of the ventral and dorsal pancreatic buds (47).

After evagination from the main endoderm tube, the pancreatic buds undergo extensive proliferation and expansion. Continuous tubular structures develop in the pancreatic progenitor domain, followed by the specification and patterning of pancreatic progenitors, resulting in formation of a bipotent “trunk” domain and multipotent “tip”

domain (48). The cells located at the trunks exhibit bipotency, and can give rise to pancreatic ductal cells or endocrine islet cells at later stages. The cells residing at the tips of the branches exhibit multipotency, and can differentiate into all the pancreatic cellular lineages including acinar cells, duct cells, and the endocrine cell lineages (48). This specification of different early pancreatic progenitor domain is also referred as “primary transition”. In the mouse pancreatic development, the primary transition spans from embryonic day (e)9.5 to e12.5 (49).

Following the primary transition, the progenitor cells continue branching into surrounding mesenchymal tissues, which provide cues for their growth and differentiation. The differentiation of pancreatic progenitor cells into acinar cells, duct cells, and endocrine cells is referred to as the “secondary transition” (49). The period of the secondary transition in mouse spans from e12.5 to birth. Although the trunk cells remain bipotent for duct and endocrine through the rest of pancreatogenesis, the tip cells lose their multipotency during the secondary transition and become limited to acinar cell differentiation (50). During this process, endocrine progenitors delaminate from the trunk region, then migrate, replicate, and differentiate into specific endocrine subtypes, and finally they aggregate into islet structures (51,52). Interestingly, for the endocrine cells, the differentiation program is not completely synchronous with exocrine pancreas development, as there is an early wave of endocrine differentiation during the primary transition stage. These early-differentiated endocrine cells primarily consist of glucagon and insulin expressing cells (53-55). Although great understanding of endocrine differentiation has been achieved within last few decades, the programming and biological functions of these early-differentiated endocrine cells still remain unknown.

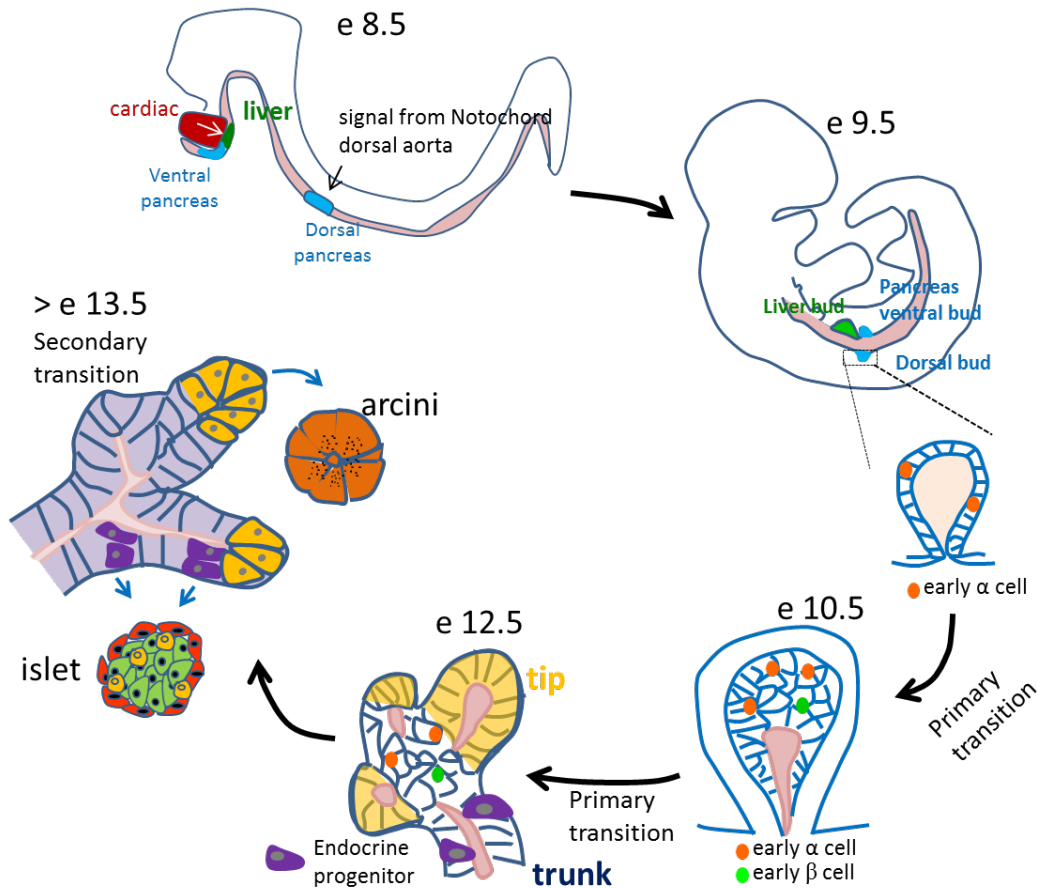


Figure 1-1. Pancreas development in the mouse. Dorsal and ventral pancreatic progenitor domains initiate in the endodermal sheet at e8.5. Signals secreted from the notochord and dorsal aorta promote dorsal pancreas formation. Signals secreted from cardiac and septum transversum mesoderm promote liver cell fate, but inhibit ventral pancreas cell fate. At e9.5 early pancreatic progenitors continue to bud from the endoderm. First wave  $\alpha$  cells differentiate in the progenitor domain. At e10.5, the pancreatic progenitor domain continues to expand. First wave  $\alpha$  and  $\beta$  cells can be detected. At e12.5, the pancreatic progenitors start to branch into the surrounding mesenchymal tissues and two distinct domains, the “tip domain” and “trunk domain,” are formed. Later (>e13.5), pancreatic progenitors start to differentiate. Acinar cells differentiate from progenitors located at the tips of the branches. Endocrine progenitors differentiate from duct structures and the five endocrine cell lineages migrate and cluster into islets. Modified from Kim, S. K. and MacDonald R.J. 2002 (56) and Zaret, K. S. 2008 (57).

From the perspective of  $\beta$  cell regeneration, the study of pancreas development can provide insight into the following questions: (1) which cells have the potential to become  $\beta$  cells and (2) how can this process be facilitated for therapeutic treatment? In general,  $\beta$  cells can be generated from stem cells or from  $\beta$  cell relatives, including the closely related endocrine cells or more distant exocrine relatives. Revealing the innate genetic programming of different pancreatic cell lineages and the extracellular signaling guiding them during pancreas development will facilitate the *in vitro* differentiation of stem cells and the *in vivo* reprogramming of  $\beta$  cell relatives.

## **ii. Innate genetic programming during pancreas development**

Each differentiated cell expresses its identity through the expression of a unique genetic program (58). The transition of progenitor cells into mature differentiated pancreatic cell types is a dynamic process that encompasses the active evolution of cellular identities. Through the use of varied powerful genetic tools including gene knock-out, gene knock-in and genetic lineage tracing, this dynamic process during pancreas development has been largely revealed. During pancreatogenesis, the key transcription factors described in sections below play important roles in defining cell identity, and loss of these results in selective impairment of pancreas maturation.

### **Pdx1 and Ptf1a: “master regulators” of the pancreas**

Pancreas and duodenal homeobox 1 (Pdx1) is considered one of the key master genes initiating the pancreas differentiation program as well as regulating mature  $\beta$  cell identity. Pdx1 is expressed in the early dorsal and ventral pancreatic progenitor domains, as well as in the duodenum and lower parts of the stomach (59). Although Pdx1 is continually expressed throughout pancreas development, its expression subsides in all but the trunk branches, which give rise to the pancreatic duct and to endocrine cells (48). High levels of Pdx1 are limited to islet  $\beta$  cells in adult pancreas (60). Knockout of Pdx1 results in complete absence of adult pancreas tissues (59). Further, in the absence of Pdx1, pancreatic progenitor expansion is arrested, and only a small number of short-lived  $\beta$ -like

cells and clusters of  $\alpha$ -like cells are evident (61). This indicates that Pdx1 is not required for the differentiation of early wave endocrine cells. The importance of Pdx1 in pancreas development has also demonstrated in human studies: mutation of *PDX1* in humans causes permanent neonatal diabetes due to pancreas agenesis (62).

Pdx1 is also crucial for  $\beta$  cell function and maintenance  $\beta$  cell identity. Conditional knockout of *Pdx1* in  $\beta$  cells impairs essential  $\beta$  cell gene expression, and leads to depletion of  $\beta$  cells and the development of diabetes (63). Molecular analyses indicate that Pdx1 binds to the promoter regions of key  $\beta$  cell functional genes where it promotes gene expression (64). Interestingly, loss of Pdx1 in  $\beta$  cells results in an increased expansion of islet  $\alpha$  cells and  $\delta$  cells at the expense of  $\beta$  cell quantity (63). On the other hand, enforcing *Pdx1* expression in pancreatic endocrine progenitors results in destabilized  $\alpha$  cell fate and reprogramming of  $\alpha$  cells into  $\beta$  cells (65,66). All these data indicate that in addition to regulating the Glucose Stimulated Insulin Secretion (GSIS) machinery in  $\beta$  cells, Pdx1 also plays a role in guiding and stabilizing pancreatic endocrine lineages during development.

Besides the important role of Pdx1, Pancreas transcription factor 1a (Ptf1a) is a second key gene that coordinates with Pdx1 to regulate pancreas progenitor fates (67,68). In contrast to *Pdx1*, the endodermal expression of Ptf1a is limited to the pancreatic progenitor domain. Lineage tracing experiments indicate that like Pdx1<sup>+</sup> cells, Ptf1a<sup>+</sup> cells in the early pancreatic progenitor domain give rise all the pancreatic cell types including acinar, duct and endocrine cells; this suggests that the expression of Ptf1a is important to maintain the multipotency of pancreatic progenitors (67). Indeed, loss of *Ptf1a* via gene knockout in mouse model results in severe impairment of pancreas development including complete absence of acinar cells (67). Furthermore, mutation of *PTF1A* in humans also causes pancreatic agenesis, which results in permanent neonatal diabetes (69).

Besides its important role in pancreatic progenitors, *Ptf1a* also acts as a master gene for maintaining acinar cell function. During the primary transition, global pancreatic Ptf1a is eventually restricted to the tip region of the pancreatic branches from which the mature acinar cells arise. In adult pancreas, the expression of *Ptf1a* is limited to acinar

cells, where it directs the transcriptional activity of secretory digestive enzyme genes (48,70). Interestingly, suppression of Ptf1a activity in differentiated pancreatic acinar cells facilitates reprogramming of acinar cells into insulin<sup>+</sup> cells (71). This evidence illustrates a level of plasticity in pancreatic cells and hints at how certain key transcription factors may play critical roles in maintaining the stability of individual pancreatic fates. As with Pdx1, the roles of Ptf1a in regulating pancreas development and acinar cellular function are highly conserved among different species (72,73).

### **Ngn3 and Neurod1: Triggers for endocrine cell differentiation**

In contrast to Pdx1 and Ptf1a, the basic helix-loop-helix (bHLH) transcription factor neurogenin3 (Ngn3) is only transiently expressed in endocrine progenitors, when they exit the cell cycle and begin differentiating into endocrine cells (74,75). *Ngn3* marks all precursors of pancreatic endocrine cells, and mice lacking *Ngn3* fail to generate any pancreatic endocrine cells and die postnatal from diabetes (74). However, although there is a complete endocrine absence in *Ngn3* knockouts, the exocrine pancreas including duct and acinar cells is relatively normal, indicating that Ngn3 plays a specific role in pancreatic endocrine lineages. Importantly, as *Ngn3* knockouts lack expression of all characteristic pancreatic endocrine genes, this indicates that Ngn3 has an important role in the initial specification of endocrine lineages from the progenitor pool (74,76). Furthermore, lineage tracing with *Ngn3-creER* has indicated that Ngn3<sup>+</sup> early progenitor cells give rise to all the endocrine lineages, but not to acinar or duct cells. This provides direct evidence that Ngn3<sup>+</sup> cells are islet progenitors (75). In mouse, there are two waves of pancreatic *Ngn3* expression. In the first, *Ngn3* is expressed from e8.5 to e11 and in the second wave, Ngn3 expression starts at ~e12.5, the initial phase of the secondary transition (53). These two waves of *Ngn3* expression coincide with the two waves of pancreatic endocrine cell formation. Although it is clear that the second wave endocrine cell formation requires *Ngn3*, the primary endocrine cells still form in the absence of Ngn3, indicating that *Ngn3* by itself is not necessary for the first wave of pancreatic endocrine differentiation. Interestingly, although *Ngn3* has a crucial role in determining endocrine lineage in mouse models, mutation of *NGN3* in humans results in a much

milder phenotype (77). While *NGN3* is expressed in human early pancreatic endocrine progenitors, persons with *NGN3* mutations are not diabetic, and present all the pancreatic endocrine cell types. This indicates that the role of *NGN3* in the regulation pancreatic endocrine cell lineages is not conserved among all vertebrates.

The *Neurogenic differentiation factor 1* (*Neurod1*) is widely expressed during development. It is first detectable in early pancreatic progenitors during the primary transition, and continues to be expressed in pancreatic  $\beta$  cells throughout development. Genetic lineage tracing has demonstrated that these early *Neurod1*<sup>+</sup> cells give rise to all the  $\beta$  cells and part of the  $\alpha$  cell population, but not to  $\delta$  cells or PP cells (78,79). Accordingly, knockout of *Neurod1* in mouse results in a significant reduction in the number of insulin producing  $\beta$  cells, failure to develop mature islets, and death shortly after birth due to severe diabetes (80). In addition,  $\alpha$  cell quantity is also reduced in *Neurod1* null mice (81). Conditional knockout of *Neurod1* in differentiated  $\beta$  cells results in a reduction of *insulin* gene expression and mis-expression of other pancreatic hormones; this indicates that *Neurod1* is required for  $\beta$  cells to achieve and maintain functional maturity (82). The critical role of *Neurod1* in  $\beta$  cell formation and maintenance is also conserved in humans. Homozygous mutation of *NEUROD1* results in permanent neonatal diabetes, and heterozygous mutations are strongly associated with T2DM (83,84). Interestingly, although *Neurod1* is a critical transcription factor regulating endocrine pancreas development, and it is not expressed in the exocrine pancreas, *Neurod1* null mouse models show impairment of exocrine pancreas and loss of acinar granules (80). These observations indicate that the development of endocrine and exocrine pancreas are connected, and that the change in the endocrine component can influence the exocrine component structure.

#### **Arx and Pax4: Antagonistic determinants of endocrine cell fate determination.**

The homeobox-containing transcription factor Aristaless related homeobox (*Arx*) is expressed throughout the genesis of the pancreas, and is subsequently expressed in islet cells. Knockout of *Arx* leads to progressive hypoglycemia, and mice die shortly after birth. Histological analysis has indicated that *Arx* mutant mice show a loss of  $\alpha$  cells and



PP cells and concomitantly, increased quantities of  $\beta$  and  $\delta$  cells; this suggests that *Arx* plays an important role in specifying  $\alpha$  cell fate during endocrine progenitor differentiation (85). Further, the forced expression of *Arx* in pancreatic progenitors, endocrine progenitors, or differentiated  $\beta$  cells drives  $\alpha$  and PP cell fates by conversion of  $\beta$  cells (86), indicating that *Arx* is necessary and sufficient for  $\alpha$  cell differentiation and inhibition of  $\beta$  cell fate. This interpretation is further supported by the observations that suppression of endogenous *Arx* is necessary to maintain  $\beta$  cell identity (87). Lastly, humans *ARX* null mutations lead to deficiency of  $\alpha$  and PP cell types, demonstrating that *ARX* has a conserved role in vertebrate pancreas development (88).

Next, the gene Paired Box 4 (*Pax4*) is expressed in the early pancreatic domain, but is later restricted to  $\beta$  cells. *Pax4* knockout results in the absence of  $\beta$  and  $\delta$  cells, but an increase of  $\alpha$  and PP cell quantity, which is opposite to *Arx* mutants (89). Further, *Arx* and *Pax4* inhibit each other at the transcriptional level, suggesting a model for early islet cell specification in which endocrine progenitors are confronted with the choice of becoming precursors of either  $\beta$  -/ $\delta$ -cells or  $\alpha$  -/PP-cells. These fates are promoted by *Pax4* and *Arx*, respectively (85,90). Finally, the heterozygous mutation of *Pax4* gene leads to early on-set diabetes, indicating that *PAX4* has a conserved role in human  $\beta$  cell development (91).

The preceding sections summarize data that have uncovered roles for certain crucial transcription factors in the determination of specific pancreatic cellular lineages. The development of the pancreas is highly regulated by the expression of these integrated transcription factor cascades. However, the delineation of this complex gene network prompts this question: how is this complex network regulated by developmental signals and changing physiological demands?

### **iii. Extracellular signals direct pancreas development**

When pancreatic progenitors bud from the endoderm, they have a multitude of differentiation potentials. During the process of pancreas development, each of these progenitor cells receives guidance from neighboring cells and in turn provides its own environmental cues. In addition to these local factors, pancreas development also

requires the integration of many systemic factors, including circulating and neuronal factors, and this environment milieu influences cellular programming. At the same time, the evolving internal programming alters cellular responses to the environmental cues. Moreover, environmental signals will continue to maintain the final differentiated identities of constituent cells so they may integratively fulfill the pancreatic functions.

During gastrulation, the embryo generates three primary germ layers: endoderm, mesoderm and ectoderm. After gastrulation, a series of morphologic movements transform the endoderm into a primitive gut tube surrounded by mesoderm. After this is established, induction of the dorsal pancreatic bud requires Fibroblast Growth Factor (Fgf) secreted from the notochord. This suppresses SHH in the endoderm and initiates *Pdx1* expression in the dorsal pancreatic progenitor domain (92,93). In addition to the notochord, transient interaction of the dorsal aorta with the pancreatic progenitor domain is crucial for the early steps of pancreas development (94). However, the molecular cues emanating from the dorsal aorta are still unclear. In contrast to dorsal pancreas induction, the induction of ventral pancreas initiation is less understood. The ventral pancreas is derived from a precursor population in a ventral endodermal region that lies near the developing heart (95). Using embryo tissue explant culture experiments, it was found that the ventral foregut endoderm defaults to a pancreatic program. However, FGF signaling from cardiac and septum transversum mesoderm diverts these progenitor to express liver genes instead of pancreas, and this occurs partially through activation of SHH signaling (95).

After the induction of the pancreatic progenitor fields, the differentiation of these cells requires signals from the surrounding mesenchymal tissues, neurons, blood vessels, and the autocrine or paracrine signals secreted from the developing pancreatic epithelium. The early interaction between pancreatic epithelium cells and surrounding mesenchymal tissues is crucial for exocrine pancreatic differentiation. The mesenchyme secretes growth factors like Fgf that induce pro-exocrine effects, and depletion of mesenchyme reveals a “default” endocrine differentiation program (96-99). Besides mesenchymal tissues, the signals secreted from islet innervating neurons also play a part in endocrine differentiation and islet architecture. For example, islet maturation and islet clustering are impaired when parasympathetic neuron innervation is blocked during development

(100). Interestingly, blood vessel invasion also influences pancreas development not only via nutrient supply, but also by endothelial signals. For example, VEGF-induced hyper-vascularization inhibits pancreas tip progenitor differentiation and decreases pancreas size (101,102). Lastly, it is clear that during development, pancreatic epithelial cells themselves secrete growth factors to self-regulate their differentiation and proliferation. For instance, TGF- $\beta$  is necessary for pancreatic duct development and restricts the progenitor differentiation toward endocrine lineages. Inhibition of TGF- $\beta$  signaling promotes endocrine cell differentiation (103,104). In contrast, EGF secreted from pancreatic epithelia promotes  $\beta$  cell formation. EGF receptor agonists in pancreatic organ cultures expand *Pdx1* expression and promote  $\beta$  cell formation at the expense of acinar cells (105). In addition to soluble growth factors, pancreatic progenitor fates are regulated through direct cellular interactions. Notch signaling, the best understood of these, mediates multiple steps of pancreas development. Mutation of Notch ligand or its downstream effectors in mice resulted in an accelerated and over-abundant commitment of the embryonic pancreatic epithelium into the endocrine lineage, suggesting that notch signaling prevents endocrine differentiation of these progenitors (106,107) and permits expansion of progenitor domain (108). Consistent with these data, Notch signaling represses *Ngn3* and *Ptf1a*, two transcription factors that determine endocrine and exocrine lineages (see above). Additionally, repression Notch signaling is thought to be required for both endocrine and exocrine pancreas differentiation (109-111). Although the Notch target gene *Hes1* is rarely expressed in adult pancreas, in certain injury models Notch signaling is reactivated, and this appears crucial for pancreas regeneration mechanisms (112-114).

### **I.C. The role of Glucagon/GLP-1 and Insulin hormones in pancreas development**

The question of how the quantities of each endocrine cell subtype are determined during development, and maintained in maturity in the face of physiological flux, is of obvious importance to understanding metabolic disease. While the final pancreas size is directly determined by the number of pancreatic progenitor cells (115), this observation still does not answer the question of how the ratio of different cell types within the

pancreas is determined. Developmental genetics studies have shown how certain transcription factors influence the balance of different endocrine subtypes, revealing that competitive expression of these transcription factors within progenitor cells may determine the final islet endocrine distribution. Yet, it still does not answer the question of how the transcription factor levels are controlled. One mechanism that balances hormone levels within the mature, functioning endocrine system is negative feedback regulation. Multiple examples of feedback regulation also happen during development, and this opens the question of whether hormonal feedback mechanisms are present during pancreatic endocrine cell development. In the following section, I focus on the two most important counterbalancing pancreatic hormones, glucagon and insulin, and discuss the current understanding of their influence on pancreas development.

#### **i. *glucagon* gene products and signaling**

Activation of the *proglucagon* gene leads to the transcription of *proglucagon* mRNA which can then be translated into proglucagon peptides. In mature islet  $\alpha$  cells, this proglucagon peptide is chiefly processed into glucagon and other fragments via the activation of prohormone processing enzyme Proprotein Convertase 2 (PC2). Besides the  $\alpha$  cells, *proglucagon* mRNA is also expressed in intestinal enteroendocrine L cells and certain neurons. However, in these non- $\alpha$  cells, the preproglucagon peptide is processed by different prohormone processing enzyme PC1. Processing of preproglucagon by PC1 leads to the production of glucagon-like peptide 1 (GLP-1), but not glucagon.

Previous studies have shown that glucagon and GLP-1 selectively bind to the Glucagon receptor (Gcgr) and GLP-1 receptor (GLP-1r), respectively, and that this activates divergent physiological functions. Affinity binding analysis indicates that high levels of Gcgr are found in the liver, adipocytes, cardiac muscle (116,117). In addition, *Gcgr* is expressed abundantly in  $\beta$  cells. Activation of Gcgr can stimulate multiple downstream signaling pathways including the cAMP-PKA pathway, elevated intracellular calcium signals, and the AMPK signaling pathway (118). Glucagon can act as local signal to regulate insulin secretion and  $\beta$  cell functions (118). Knock-out of *Gcgr* in mice results in reduced insulin secretion in response to glucose (119). Conversely,

over-expression of Gcgr in  $\beta$  cells promotes insulin secretion and increased  $\beta$  cell mass, indicating that glucagon signaling exhibits a positive role in  $\beta$  cell function (120).

GLP-1 belongs to the incretin class of hormones that lower glucose and stimulate insulin secretion (121) and the physiological functions of GLP-1 are quite different from glucagon. In some regards, it elicits physiological actions opposite to glucagon. The biological functions of GLP-1 include GSIS stimulation, increase of insulin biosynthesis, and inhibition of glucagon secretion, (121). Within in the pancreas, GLP-1r is highly expressed in  $\beta$  cells and duct tissues (122). In  $\alpha$  cells, GLP-1r is highly expressed during fetal development, but is decreased by adulthood (123), though a subpopulation of  $\alpha$  cells may retain weak expression of GLP-1r in adults (122). Besides the endocrine pancreas, GLP-1r is also weakly expressed in acinar cells. The direct effects of GLP-1 on  $\beta$  cell insulin secretion are unclear, despite much study (124). As with the Gcgr, GLP-1r is a G-protein coupled receptor. Activation of GLP-1r drives cAMP production and activates the downstream PKA signaling pathway. PKA then directly acts on insulin secretory machinery, or indirectly promotes insulin secretion by elevating intracellular calcium levels. In addition, treatment with the GLP-1r agonist Exendin-4 in adults can increase  $\beta$  cell mass (125). Finally, activation of the GLP-1r stimulates the PI3K and PKA pathways to drive proliferative and anti-apoptotic effects in  $\beta$  cells (126,127).

## **ii. *Insulin* gene products and signaling**

As with GLP-1, insulin is also processed by the prohormone peptide preproinsulin by protein convertase 1 (PC1). In all vertebrates, insulin is mainly produced by the  $\beta$  cells, with some neural production as well (128,129). Insulin secreted from the  $\beta$  cells is critical for maintaining metabolic homeostasis. *Insulin receptor (Insr)* is ubiquitously expressed in most cell types, but exhibits highest levels in metabolically active tissues like liver, muscle and adipocytes (130). In peripheral metabolic tissues, activation of Insr stimulates glucose uptake and energy storage (131). Insulin signaling also plays crucial roles in regulating embryonic development and tissue growth. Activation of Insr induces phosphorylation of its major substrates Insulin Receptor Substrate 1 (IRS1) and -2 (IRS2), which then stimulate downstream pathways. These include PI3K-Akt and Ras-

Raf-MAPK pathways (132). Insulin signaling regulates gene expression through Foxo proteins, a subclass of the fork-head transcription factor family. Activation of PI3K-Akt via insulin stimulation can shuttle Foxo out of the nucleus, thus relieving Foxo-mediated repression of insulin target genes (133).

Within the pancreas, *Insr* is expressed in islet cells, and Insulin signaling has crucial roles in regulating islet hormone secretion. Both *in vitro* and *in vivo* studies have shown that glucose-regulated glucagon secretion requires *Insr* expression in  $\alpha$  cells (134,135). Moreover, knockout of *Insr* in  $\beta$  cells impairs insulin secretion in response to glucose stimulation, and leads to progressive loss of glucose tolerance (136). These data indicate that insulin acts as an autocrine and paracrine factor to regulate islet functions. Besides endocrine components, it is also shown that insulin receptor also expressed in pancreatic duct cells and acinar cells (137). Activation of *Insr* is important for acinar cell functions: it regulates digestive enzyme biosynthesis and secretion (138).

### **iii. Glucagon/GLP-1 and Insulin regulate pancreas development**

Although the vast majority of Glucagon/GLP-1 and Insulin signaling research has focused on their physiological roles in metabolism, some limited data have shown that these two signals may also have important roles in pancreas development. In rodents, disruption of glucagon signaling not only results in hypoglycemia as expected, but also induced significant changes in pancreas and islet phenotypes. Loss of glucagon signaling produces a dramatic expansion of  $\alpha$  cell mass, but no significant change in  $\beta$  cells (139). Interestingly, many  $\alpha$  cells that develop in the absence of Glucagon mis-express  $\beta$  cell-specific markers, like Pdx1 and Glut2. Although there is no significant change of  $\beta$  cell quantity in *Gcgr*<sup>-/-</sup> adult mice,  $\beta$  cell functions and expression of key  $\beta$  cell genes is significantly reduced (140). This indicates that Glucagon is involved in  $\beta$  cell maturation. Moreover, studies using *in vitro* pancreatic explant cultures have indicated that Glucagon signaling is required for the induction of early  $\beta$  cells (141). These findings indicate that Glucagon can stimulate  $\beta$  cell differentiation and insulin expression. Moreover, increased calcium influx also increases *insulin* promoter activity, which may also partially explain Glucagon-induced *insulin* gene transcription (142). Together, these

results indicate that Glucagon exhibits  $\beta$  cell-promoting activities during development. However, the mechanisms of glucagon-regulated  $\beta$  cell generation are still unclear.

There is accumulating evidence that GLP-1 signaling protects against  $\beta$  cell injury and promotes  $\beta$  cell regeneration, but there is little evidence for a role for GLP-1 during pancreas and  $\beta$  cell development. Knockout *GLP-1r* in mice results in impaired glucose responses in  $\beta$  cells, but no significant morphological changes in the pancreas and islet (143). However, studies of *in vitro*  $\beta$  cell differentiation show that exendin-4 treatment promotes early induction of  $\beta$  cells from pancreatic progenitors (144). In adult pancreas, *GLP-1r* is highly expressed in  $\beta$  cells and a subpopulation of pancreatic duct cells (122). However, the expression pattern of *GLP-1r* in fetal pancreas is not clear. Furthermore, previous studies have identified a population of adult pancreatic duct cells that exhibit a potential for  $\beta$  cell differentiation, and this can be accelerated by GLP-1r activation (125). This suggests that GLP-1 acts locally to regulate  $\beta$  cell development, and this is underscored by the *GLP-1r* knockout studies (145).

In spite of the clear evidence for insulin signaling in regulating metabolism, little is known about the role of Insulin in regulating pancreas development and  $\beta$  cell neogenesis. It has been shown that in embryonic stem cells (ESC), insulin signaling is needed for self-maintenance; blocking insulin signaling promotes differentiation of ESC (146). Similar phenomena have been seen in hematopoietic stem cells and adult intestinal stem cells (147,148). Together, these studies indicate that the organ development is tightly linked with metabolism. Thus, pancreatic progenitor expansion and differentiation are likely to be regulated by Insulin, as it is a key nutrient sensor. Indeed, abundant evidence shows that Insulin signaling is required for  $\beta$  cell compensation and glucose homeostasis in adults. *Insr* is globally expressed in both endocrine and exocrine pancreatic compartments, and much interest has focused on the autocrine effects of insulin signaling in regulating  $\beta$  cell proliferation. For instance, knockout of *Insr* in  $\beta$  cells impairs glucagon tolerance and results in failure of  $\beta$  cell compensation due to the blockage of  $\beta$  cell proliferation (136).

The autocrine stimulation of insulin signaling in  $\beta$  cells plays an important role in facilitating Pdx1 expression and normal  $\beta$  cell function by controlling Foxo transcription

factor localization (149). Interestingly, knockout of *Foxo* genes in  $\beta$  cells leads to loss of  $\beta$  cell character and induces dedifferentiation of  $\beta$  cells (150). Further, although Pdx1 is inhibited by Foxo expression, expression of other  $\beta$  cell transcription factors such as *Neurod* and *Mafa* are enhanced upon Foxo activation (151). The complex role of *Foxo* genes in regulating metabolism and  $\beta$  cell fate may reflect the mixed role of insulin signaling in regulating  $\beta$  cell differentiation and growth.

In addition to its autocrine effects, insulin signaling also acts as important paracrine regulator, mediating other islet cell functions, such as  $\alpha$  cells (135). Moreover, it can stimulate increased proliferation of pancreatic duct cells (152). During embryonic development, pancreatic duct epithelial cells exhibit a potency to differentiate into endocrine cells, including  $\beta$  cells. Thus, understanding the roles of insulin signaling in pancreatic development will likely have a strong impact on research into mechanism of  $\beta$  cell regeneration.

#### **I.D. *In vivo* $\beta$ cell regeneration from endogenous reservoirs**

From discussion above, we have shown that studies of pancreas development have revealed genes and signals that promote specific pancreatic cell lineages, and these have been effectively manipulated to promote both *in vitro* and *in vivo*  $\beta$  cell regeneration. Recent breakthroughs have been made in this field for producing glucose responsive insulin secreting  $\beta$  cells *in vitro* from human stem cells. Transplantation of these *in-vitro* differentiated  $\beta$  cells are able protect against hyperglycemia in Diabetic mouse models (24,26). Although great advances have been made in the research of *in vitro*  $\beta$  cell production, there are still great ethical and social hurdles to be overcome before these stem cell therapies can move forward for the treatment of diabetic patients. However, previous human and animal studies have shown evidence for the existence of spontaneous endogenous  $\beta$  cell regeneration *in vivo*. Moreover, recent studies have shown that besides proliferation of existing  $\beta$  cells,  $\beta$  cell can also regenerate from non- $\beta$  cell sources, and has suggested that there are great reservoirs for endogenous  $\beta$  cell regeneration via neogenesis. However, much remains to be learned about the molecular mechanisms that regulate  $\beta$  neogenesis.



### **i. Cellular sources of endogenous $\beta$ cell neogenesis**

The study of pancreas development has revealed how internal and external signals direct the progenitors to their final differentiated cell fates. Recent findings indicate that alteration of these internal or external cues can dramatically influence progenitor cell fate specification or even transform one differentiated cellular fate into another. Excitingly, the plasticity of pancreatic cells, revealed from developmental studies, has opened new directions for study in the *in vivo*  $\beta$  cell regeneration field, as it is possible for many other pancreatic cell types to be transformed into  $\beta$  cells. So far, the pancreatic  $\alpha$  cells,  $\delta$  cells, acinar cells, and duct cells all have been shown to have some potential to serve as new cellular sources for *in vivo*  $\beta$  cell regeneration.

### **The intertwined fates of $\alpha$ and $\beta$ cells**

From a physiological perspective, glucagon-secreting  $\alpha$  cells and insulin-secreting  $\beta$  cells execute opposite, complementary biological functions.  $\beta$  cells clear blood glucose and promote energy storage while glucagon mobilizes the stored nutrition into useable energy forms (131). This yin-yang balance between  $\alpha$  and  $\beta$  cells is crucial to maintain balanced metabolism. Pancreas developmental studies have revealed that these two cell lineages are derived from the same progenitors (TrPCs), and can interconvert under certain conditions. This inter-conversion can be mediated by the loss or gain of any of several key transcription factors. For instance, over-expression of *Pax4* in pancreatic or endocrine progenitors drives conversion of  $\alpha$  cells into  $\beta$  cells; this in turn drives new  $\alpha$  cell differentiation to replace the lost cells. These newly formed  $\alpha$  cells are also eventually transformed into  $\beta$  cells, resulting in  $\beta$  cell hyperplasia and hypoglycemia (153). In addition, mis-expression of *Pdx1* in endocrine progenitors induces the transformation of  $\alpha$  cells into  $\beta$  cells during a developmental window (65). This  $\alpha$  to  $\beta$  cell reprogramming can also be induced by loss of key  $\alpha$  cell transcription factors, like *Arx*. Knockdown of *Arx* results in a complete loss  $\alpha$  cells together with increased  $\beta$  cell populations (85). Specific deletion of *Arx* in  $\alpha$  cells triggers their transformation into  $\beta$  cells (154). On the other hand, enforcing expression of *Arx* in endocrine progenitor results in a drastic loss of  $\beta$  cells and concurrently increases  $\alpha$  cells (86). The

transformation between  $\alpha$  cells and  $\beta$  cells is likely due to their close developmental relationship in that they share a common ancestor.

Using different approaches, the epigenetic and transcriptional landscape of human pancreatic  $\alpha$  cells,  $\beta$  cells, and exocrine cells have been analyzed by large-scale ChIP sequencing and RNA sequencing. These studies showed that many  $\beta$  cell specific genes are not fully repressed in  $\alpha$  cells, but rather are epigenetically marked as “bivalent”, meaning that these genes can be rapidly induced, permitting  $\alpha$  to  $\beta$  cell transformations under certain conditions (155). However, neither  $\beta$  cells, nor exocrine cells exhibit this pattern of bivalent epigenetic markers. This evidence indicates that  $\alpha$  cells have special character, and that the barriers for  $\alpha$  to  $\beta$  cell reprogramming may be lower than reprogramming from other pancreatic cell types. It also suggests that  $\alpha$  cells exhibit certain  $\beta$ -progenitor characteristics. This hypothesis is also supported by the evidence that dual hormone-expressing cells, which co-express insulin and glucagon, can be broadly detected in the fetal pancreas, but are rarely detected in adults (156-158). However, this hypothesis is challenged by lineage tracing experiments, which indicated that  $\beta$  cells never arise from  $\alpha$  cells, and vice-versa (159). Thus, whether  $\beta$  cells are derived  $\alpha$ -like progenitors during development is controversial.

Transformation of  $\alpha$  cells to  $\beta$  cells has also been shown to occur during  $\beta$  cell regeneration. In a mouse model permitting specific, extreme  $\beta$  cell ablation (>99%),  $\beta$  cells regeneration was observed. Further, genetic lineage tracing showed that a portion of the newly regenerated  $\beta$  cells were derived from pre-existing glucagon<sup>+</sup>  $\alpha$  cells (160). The  $\alpha$  to  $\beta$  cell transformation was also detected in another rodent study using a combination of genetic and chemically-induced  $\beta$  cell ablation (161). Interestingly, in other  $\beta$  cell ablation mouse models, in which less severe  $\beta$  cell ablation was performed,  $\beta$  cell regeneration was mediated by self-replication rather than neogenesis (162). Thus it is clear that the injury model influences the mode of  $\beta$  cell regeneration, but the mechanisms regulating this are unknown. Furthermore, it is completely unknown which endogenous cues might control  $\beta$  cell regeneration from different cellular sources. Unveiling these endogenous factors will certainly lead to new drug targets for diabetes treatments that aim to restore functional  $\beta$  cell mass.

Interestingly, when compared with  $\beta$  cells,  $\alpha$  cells exhibit a much greater regenerative potential. When  $\alpha$  to  $\beta$  cell transformation was induced by either *Pax4* mis-expression or *Arx* knockout, the converted  $\alpha$  cells were rapidly replenished by  $\alpha$  cell neogenesis from pancreatic duct cells (153,154). Conversely, no analogous  $\beta$  cell neogenesis was detected after *Arx* expression in endocrine progenitors was used to drive  $\beta$ -to- $\alpha$  transformation (86). Furthermore,  $\alpha$  cell hyperplasia, via increased  $\alpha$  cell proliferation and neogenesis, has been observed after knockdown of *glucagon receptor* or *PC2* (139,163). The molecular signaling cues that drive  $\alpha$  cell hyperplasia under conditions of glucagon signaling deficiency are unknown, but circulating factor(s) derived from the liver may be involved (164). During the progression of both Type 1 and Type 2 diabetes, increased  $\alpha$  cell proliferation has been observed together with the decrease of  $\beta$  cell mass (165). Thus, understanding the relationships between  $\alpha$  and  $\beta$  cells under normal and pathological conditions, and uncovering the molecular cues that regulate  $\alpha$  to  $\beta$  transformation, may help to re-establish metabolic homeostasis in diabetic patients.

### **A new appreciation for $\delta$ cells**

In addition to  $\alpha$  cells, the somatostatin<sup>+</sup> delta ( $\delta$ ) cells exhibit some plasticity. Somatostatin plays an important role as a local inhibitory factor to repress insulin and glucagon secretion in the islet (166). Besides this local action, release of somatostatin from the islet plays an important role in regulating exocrine pancreas and GI tract functions (167).  $\delta$  cells share expression of some transcription factors with  $\beta$  cells, including *Pax4* and *Pdx1* (168,169), implying a relationship  $\beta$  cells and  $\delta$  cells that is supported by some genetic studies. For instance, knockout of *Arx* in the pancreas increases both  $\beta$  and  $\delta$  cell populations (85), while misexpression of *Arx* induces loss of both  $\beta$  and  $\delta$  cells (153). However, the mechanisms mediating differential specification of  $\delta$  and  $\beta$  cells is still unclear.

Recently, it was found that  $\beta$  cell injury in young mice triggered  $\delta$  cell, but not  $\alpha$  cell, transdifferentiation into  $\beta$  cells. However, this capacity for conversion was lost with aging, while age only increased  $\alpha$  cell plasticity (170). The transformation of  $\delta$  to  $\beta$  cells involves first the proliferation of  $\delta$  cells, and then dedifferentiation. This proceeds to

*Ngn3* re-expression and re-differentiate into Insulin<sup>+</sup> cells and Somatostatin<sup>+</sup> cells. As such, it is likely that  $\delta$  cell characteristics, and their response to  $\beta$  cell loss, are very different at juvenile and adult stages. Uncovering the molecular mechanisms that are involved in juvenile  $\delta$  cell adaptability will shed further light on islet cell plasticity and  $\beta$  cell regeneration.

### **Duct cells: Progenitors hiding in plain sight?**

The potential of pancreatic ductal cells as sources of  $\beta$  cell regeneration has been a long-debated and controversial topic. This debate has centered on the questions of 1) Whether pancreatic progenitor cells exist in or adjacent to the adult pancreatic duct, and 2) Whether this cellular source is relevant to islet regeneration.

During development, duct-like epithelial cells in the trunk of the pancreatic arbor (trunk progenitor cells; TrPCs) are the precursors of endocrine cells. *Ngn3*<sup>+</sup> TrPCs delaminate from the epithelium and subsequently differentiate into endocrine cells (44). However, it is unclear whether pancreatic duct remains competent for endocrine neogenesis in the adult pancreas. Largely circumstantial evidence suggests that it does. For instance, histological analyses of adult pancreatic tissues in both human and rodent samples show Insulin<sup>+</sup> cells within the duct epithelium, and small clusters of endocrine cells budding from it (171). Furthermore, budding of  $\beta$  cells from duct epithelium region and proliferation of pancreatic duct cells were found to be increased in certain  $\beta$  cell injury models such as partial pancreatectomy (172). Moreover, the long-half life and slow turn-over of  $\beta$  cells suggest  $\beta$  cell replication may not be sufficient to explain the  $\beta$  cell expansion seen after birth or during  $\beta$  cell compensation (173,174). In certain human pathologies, diffuse islet cell hyperplasia called nesidioblastosis apparently arises from the duct epithelium and is associated with hyperinsulinemia and hypoglycemia; this is often diagnosed in children but also reported in adults (175).

The hypothesis that duct cells can serve as an adult  $\beta$  cell progenitor pool is further supported by some genetic evidence. First,  $\beta$  cell regeneration studies that use the partial duct ligation (PDL) model in rodents (176) showed that *Ngn3* was expressed in ducts following PDL in adult mice, and lineage tracing and transplantation assays showed that

these *Ngn3*<sup>+</sup> cells could differentiate into Insulin<sup>+</sup>  $\beta$  cells. Secondly, lineage tracing results using the pancreatic duct-specific promoter carbonic anhydrase II (*cpaII*) showed that pancreatic progenitor cells residing in the pancreatic ducts give rise to both endocrine and exocrine components after birth (177). These exciting studies suggest that pancreatic duct cells retain a “developmental memory” that can be replayed for new  $\beta$  cell generation; However, some conclusions derived from these studies have been subsequently challenged. Multiple studies using  $\beta$  cell-specific and duct-specific genetic lineage tracing tools failed to detect significant  $\beta$  cell neogenesis from the duct or any other sources (178-180). These studies support the conclusion that duct cells give rise to  $\beta$  cells only during pancreatogenesis. However, it is important to recognize that these studies underscore the difficulty in detecting neogenesis against a background of pre-existing  $\beta$  cells and substantially altered exocrine pancreatic mass. Therefore, it is still controversial whether the pancreatic duct cells can serve as a significant reservoir for  $\beta$  cell generation in adults.

### **Acinar cells: how to shorten the distance to $\beta$ cells?**

From a developmental perspective, the acinar cells are perhaps the most distant pancreatic relative of  $\beta$  cells. Acinar cell differentiation is initiated at the tip of the branched pancreatic arbor by signals from adjacent mesenchyme during the primary transition (181). Interestingly, the tip progenitor cells (TipPCs) remain multipotent throughout early embryonic stages, retaining the ability to differentiate into duct and endocrine cells as well as acinar cells (48). Whether acinar cells can be experimentally converted into  $\beta$  cells has been the focus of much research.

The first successful attempts to transform acinar cells into  $\beta$  cells proceeded to activate  $\beta$  cell fate through the expression of three key  $\beta$  cell developmental regulators. Using adenovirus transfection, the combination of *Ngn3*, *Mafa* and *Pdx1* in acinar cells transformed them into insulin-expressing cells, which could ameliorate STZ-induced hyperglycemia (182). Besides over-expression of  $\beta$  cell regulators, the loss of Ptf1a activity is also sufficient to transform acinar cells into  $\beta$  cells (183). Despite these limited successes, the potential of acinar to  $\beta$  cell transformation has only been detected in cases

of genetic manipulation; no spontaneous examples of regenerative transdifferentiation have been reported *in vivo*. Even so, in some pathologies, such as pancreatic cancer acinar cells are well known to transdifferentiate into duct epithelium cells, and this process may involve the dedifferentiation of acinar cells (184). Thus, understanding the mechanism of acinar-to-duct transformation may help reveal a path from acinar cells to  $\beta$  cells.

## **ii. Signals regulating pancreatic $\beta$ cell development promote *in vivo* $\beta$ cell regeneration**

$\beta$  cell regeneration may recapitulate certain aspects of development, using similar signals and mechanisms to drive the regeneration process. For example, Activin, an important extracellular signal that regulates pancreatic progenitor initiation and endocrine cell development was found to be up-regulated in adult pancreatic ducts upon STZ-induced  $\beta$  cell ablation (185). Administration of Activin in neonatal STZ-treated rats can increase  $\beta$  cell regeneration and improve glucose homeostasis (186). Besides Activin, other growth factors, including EGF, FGF, and growth hormone, which regulate  $\beta$  cell development also play positive roles promoting  $\beta$  cell regeneration *in vivo* through  $\beta$  cell proliferation (187).

In addition to growth factors and hormones, cytokines and inflammatory factors also contribute to  $\beta$  cell regeneration (188-190). After  $\beta$  cell injury, the induction of an immune response to remove dead cells promotes the remodeling of existing pancreas/islet tissue. This immune response is highly dependent on the injury mechanism, and is likely to affect the mode of  $\beta$  cell regeneration in different animal models. For example, surgical injury, i.e., partial pancreatectomy, induces  $\beta$  cell proliferation and some potentially some neogenesis from pancreatic ducts (171). However, after PDL, severe pancreas remodeling occurs, together with a more robust immune response. The unique inflammatory environment resulting from PDL is an essential trigger for pancreatic duct cells to activate *Ng2* expression. Lastly, the transient induction of vascular endothelial growth factor-A (VEGF-A) expression in  $\beta$  cells increases intra-islet endothelial cells and induces  $\beta$  cell loss. Return to basal intra-islet VEGF levels promotes  $\beta$  cell regeneration,

and bone marrow derived macrophage cells are shown to be critical for this process (191).

Although these studies contribute much to the understanding of  $\beta$  cell regeneration, the complete extracellular milieu of signaling cues that guide different cellular sources during their responses to  $\beta$  cell injury is not known. Additionally, despite the fact that spontaneous transformations of cellular identity are observed in some  $\beta$  cell injury models, the endogenous triggers of this transformation are wholly unknown. Moreover, two or more cellular sources may contribute new  $\beta$  cells in a given injury model. It is not clear how these different cellular sources are coordinated. Certainly, a clearer understanding of the extracellular signaling pathways controlling *in vivo*  $\beta$  cell regeneration will provide new drug targets for innovative diabetes treatments.

### **iii. Mammalian models used to study endogenous $\beta$ cell regeneration**

In order to find therapeutic targets to stimulate *in vivo*  $\beta$  cell regeneration, numerous animal models have been developed to mimic human diabetes. These animal studies can facilitate understanding of the mechanisms of innate  $\beta$  cell regeneration and how diabetic pathology influences  $\beta$  cell regeneration. Results from these animal studies have revealed that there are two modes of  $\beta$  cell regeneration:  $\beta$  cell replication and  $\beta$  cell neogenesis (192,193). The latter involves the formation of new  $\beta$  cells from cellular sources that do not express insulin, including facultative progenitor cells or differentiated non- $\beta$  cells. Furthermore, the regeneration mode is dependent on age, injury types, genetic background and animal species. Importantly, the mode of pancreatic injury strongly influences the mode of pancreatic repair. The animal models used to study  $\beta$  cell regeneration can be classified into four categories based on injury type: (1) chemically-induced  $\beta$  cell injury, (2) surgical pancreatic injury, (3) transgene-mediated  $\beta$  cell injury and (4) induction of insulin resistance. Although the majority of  $\beta$  cell regeneration studies have been performed in rodent models, recent studies, including those in this dissertation, have revealed that zebrafish can also be used as a powerful model for diabetes and  $\beta$  cell regeneration.

### **Chemically-induced $\beta$ cell injury model**

Alloxan and streptozotocin (STZ) are two chemical compounds that are commonly used to induce  $\beta$  cell ablation. Both are glucose analogs, and specifically accumulate in  $\beta$  cells after entering via the *Glut2* transporter. Alloxan induces  $\beta$  cell apoptosis/necrosis through a localized increase in reactive oxygen species (ROS), while STZ induces  $\beta$  cell apoptosis through DNA alkylation (194). Due to its less toxic side effects, STZ is more widely used. It was first reported in the 1970s that STZ induced  $\beta$  cell destruction and could be used to mimic T1DM. Since that publication, myriad publications have investigated  $\beta$  cell regeneration after STZ-induced  $\beta$  cell loss (195). The detailed mechanism of  $\beta$  cell destruction after STZ treatment is still a matter of debate, but is dependent on the dose of STZ and age of treated animal. Both  $\beta$  cell replication and  $\beta$  cell neogenesis mechanisms have been reported during islet recovery using this model (196,197).

### **Surgical injury models**

Two main surgical models have been commonly used for  $\beta$  cell regeneration studies: pancreatectomy (PX), in which portions of the pancreas are removed, and partial duct ligation (PDL), in which a portion of the pancreatic duct is ligated to trigger acinar cell autolysis. After 50%-70% PX, rodents can maintain normal unchallenged blood glucose levels, but exhibit impaired glucose tolerance; obvious fasting hyperglycemia develops only after >90% PX (198). Interestingly, increased  $\beta$  cell replication and  $\beta$  cell neogenesis are found to be initiated at 50% PX, indicating hyperglycemia itself is not solely the  $\beta$  cell regenerating trigger (199,200). Interestingly, although obvious  $\beta$  cell regeneration happens in rodent models, PX in adult humans does not provoke  $\beta$  cell replication, but it does induce hyperglycemia and diabetes (201). These data indicate that rodents exhibit greater adaptation to their physiological environment than humans, and also a greater  $\beta$  cell regeneration capacity. In contrast to PX, an aggressive inflammatory response is induced following PDL, together with substantial acinar cell apoptosis and depletion (202,203). It has been proposed that the infiltration of immune cells and the inflammatory environment influence cellular behavior of surviving exocrine ductal tissue



and endocrine cells. Further, PDL can activate the pancreatic progenitor program in duct cells and these progenitor cells can further differentiated into  $\beta$  cells (see above) (172,176).

### **Transgenic models of $\beta$ cell injury and regeneration**

In order to more specifically induce  $\beta$  cell destruction in a temporally-controllable manner, several transgenic rodent models have been developed. In these, expression of toxic-genes is induced in  $\beta$  cells via the control of either the *Insulin* or *Pdx1* promoter. The first transgenic model that was derived by this strategy was *Tg(ins:INF- $\gamma$ )*, which was shown to mimic the islet destruction and lymphocyte infiltration observed in Type 1 diabetes (204). Increased duct epithelial cell proliferation was observed in this model, and new islets were formed near the ducts. In a second model, temporally-controlled repression of  $\beta$  cell function was exerted via suppression of *Pdx1* in  $\beta$  cells (205). In this system, *Tg(Pdx1<sup>rtTA</sup>)Tg(Tet-Pdx1)*, *Pdx1* was specifically dampened by administration of doxycycline; withdraw of doxycycline drove  $\beta$  cell formation adjacent to pancreatic ducts. In a third approach, diphtheria toxin A (DTA) was used to induce specific  $\beta$  cell death. In these *Tg(Ins:rtTA, Tet:DTA)* mice, about 50%-70%  $\beta$  cells were destroyed following administration of doxycycline (162). Lineage tracing revealed that  $\beta$  cell replication provided all of the regenerated  $\beta$  cells observed in this model. However, in another transgenic model based on misexpression of the human diphtheria toxin receptor, *Tg(Ins:DTR)* mice can present with destruction of 99%  $\beta$  cells after diphtheria toxin administration, and  $\beta$  cell regeneration can be observed afterward it's clearance (160). Lineage tracing demonstrated that most regenerated  $\beta$  cells in this model arise by  $\alpha$  cell transdifferentiation. Fourthly, the inducible and reversible ablation of pancreatic  $\beta$  cells can be achieved in the PANIC-ATTAC model, whereby efficient  $\beta$  cell apoptosis can be driven via conditional misexpression of Caspase 8 (206). Finally,  $\beta$  cell regeneration studies have been carried in mutant mice that mimic the development of both T1DM and T2DM, such as AKITA and NOD mice and ZDF rats (207,208). Together, these rodent models have shown that  $\beta$  cells regenerate from multiple cellular sources, and that the mode of  $\beta$  cell regeneration is highly dependent on the injury mode. However, the

triggering factor(s) that activate and coordinate the different cellular contributions to new  $\beta$  cell regeneration are entirely unknown.

### **Regeneration by $\beta$ cell compensation in models of insulin resistance**

Insulin resistance in T2DM can result increased  $\beta$  cell mass (209). Although the mechanisms mediating this expansion are still unclear, it has been proposed to involve a reduced response to insulin in target tissues and in the islet itself. However, knockout of *Insulin Receptor (Insr)* in most energy reservoirs like adipose or skeletal muscle does not increase  $\beta$  cell mass, and compensatory  $\beta$  cell regeneration is only observed in liver-specific *Insr* knockout mice (210-214). The fact that only liver-*Insr* knockouts induce compensatory  $\beta$  cell mass expansion suggests a feedback regulatory mechanism between liver and pancreatic endocrine cells. Subsequent studies showed that this feedback regulation was mediated by circulating factors derived from the liver, which are suppressed by insulin signaling (215).

### **I.E. Zebrafish as a model for pancreas development and $\beta$ cell regeneration**

Although the vast majority of animal models used to study pancreas development and  $\beta$  cell regeneration are rodents, other organisms have emerged as suitable alternative systems, and are actively being used. The zebrafish (*Danio rerio*), a tropical fish originally from the Ganges flood plains of India, has become a powerful alternative for biomedical and genetic studies (216). One advantage of this system is that zebrafish are especially genetically amenable, which makes *in vivo* large-scale mutagenesis and screening strategies possible. Importantly, zebrafish share a considerable amount of genetic identity with humans, with almost 70% of human genes having at least one zebrafish orthologue (217). In addition, its transparent body makes zebrafish an excellent tool for *in vivo*, noninvasive, imaging studies. Thus, this small vertebrate model represents a valuable tool in many areas of investigation (218). Crucially, the pancreatic developmental program and metabolic profile are highly conserved between mammals and zebrafish (216,219-221). In this dissertation, I have used varied cellular and genetic

approaches in zebrafish to investigate the hormonal control of pancreas development as well as  $\beta$  cell regeneration.

### **i. Pancreas development in zebrafish**

The zebrafish pancreas comprises both endocrine and exocrine components. There are four types of endocrine cells known in zebrafish pancreas, including insulin-secreting  $\beta$  cells, glucagon-secreting  $\alpha$  cells, somatostatin-secreting  $\delta$  cells, and rare ghrelin-secreting  $\epsilon$  cells. Moreover, the zebrafish exocrine pancreas is composed of acinar cells, pancreatic duct cells, and centroacinar cells. Digestive enzymes, including elastase and carboxypeptidases, are secreted from acinar cells and enter the intestinal bulb via the pancreatic ductal system (220).

A diagram of zebrafish pancreas development is shown in Figure1-2. As in mammals, the zebrafish pancreas is generated from dorsal and ventral pancreatic progenitor domains (220). Specification of the dorsal bud, which gives rise only to endocrine cells (222), occurs at gastrulation, commensurate with endoderm formation. At 14 hours post fertilization (hpf), the dorsal progenitors differentiation: the first endocrine cells to appear are  $\beta$  cells, followed by  $\alpha$  cells then  $\delta$  cells. These early differentiated endocrine cells aggregate to form an islet by 24 hpf. Interestingly, it is likely that these early derived zebrafish dorsal endocrine cells represent a conserved population analogous to the mammalian first wave endocrine cells. The ventral pancreas progenitors are specified from ventral endoderm around 31 hpf. After evaginating, these progenitors migrate toward, and then engulf, the dorsal endocrine cells. The ventral bud differentiates into both endocrine and exocrine cells. During the later larval period (>5 dpf), endocrine cells continually differentiate from endocrine progenitors that reside throughout the pancreatic duct network. These ventral bud-derived endocrine cells migrate to join the dorsal bud-derived endocrine cells and form one “principal islet”, which is located in the pancreatic head, as well as nucleating new islets at late larval and juvenile stages (222). The zebrafish exocrine pancreas cytodifferentiation begins around 3 dpf, and clear zymogen granules are evident in acinar cells by 84 hpf (223). Formation of the functional exocrine pancreatic digestive system is necessary for the transition from

embryonic to larval stages, since after 5 dpf, the maternally provided yolk is exhausted and the larva requires external feeding. Switching nutrient supply stimulates secondary islets to form in body of the pancreas (2,224), which differentiate from intra-pancreatic duct associated progenitor cells (225).

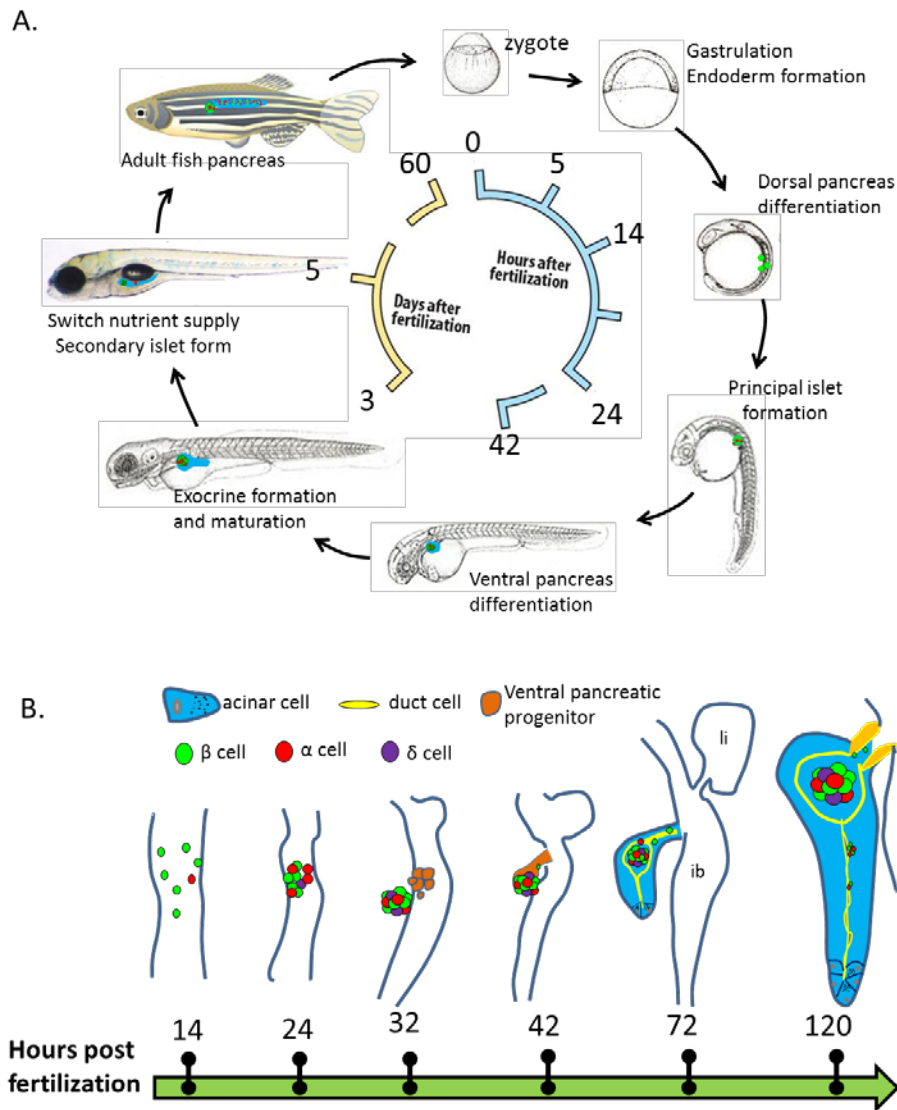


Figure 1-2. Pancreas development in zebrafish. (A) Pancreatogenesis shown within the time frame of embryogenesis and pancreas development.  $\beta$  cells are represented by green,  $\alpha$  cells by red, and exocrine pancreas by blue. (B) Morphology of zebrafish pancreas development. At 14 hpf, pancreatic dorsal bud begins differentiation into endocrine cells; these cluster into the principal islet by 24 hpf. At 32 hpf, ventral pancreatic progenitors initiate in endoderm. By 42 hpf, ventral progenitors have fused with the islet. Endocrine cells continually differentiate from ventral progenitors and migrate to the principal islet. By 72 hpf, the growing pancreas has formed a head and tail region. Acinar cells begin cytodifferentiation. By 120 hpf, secondary islets start to form in the pancreas tail.

Lineage tracing has shown that the progenitor cells in pancreatic duct region are multipotent, and give rise to endocrine and acinar cell lineages. Moreover, these progenitors exist throughout adult stages in zebrafish, indicating that zebrafish pancreas may exhibit higher regeneration capacity compared with mammals (2).

## ii. Conserved genetic programming in zebrafish pancreas development

Not only are the key steps in the pancreas development conserved between zebrafish and mammals, but the genetic developmental program is conserved as well. The key transcription factors in zebrafish pancreatic development are shown in Figure 1-3.

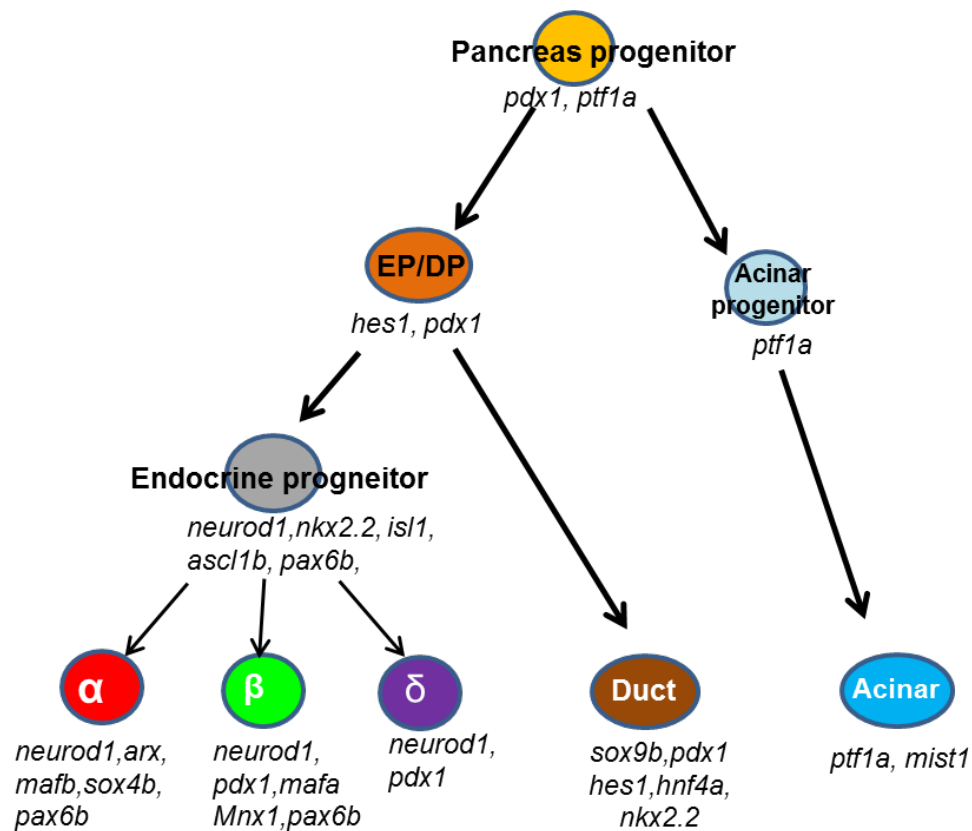


Figure 1-3. Diagram of key transcription factors in regulating zebrafish pancreas development. EP/DP: bi-potent progenitors which gives rise to both endocrine cells and duct cells. Hes1: target genes involved in regulating Notch signaling pathway.

As in mammals, Pdx1 and Ptf1a play important roles in defining zebrafish pancreatic progenitors, as well as in regulating pancreatic progenitor differentiation. The expression of *pdx1* can be detected in the dorsal bud progenitors at 12 hpf. At 24 hpf, *pdx1* is mainly expressed in  $\beta$  cells, and upon the onset of ventral bud specification, *pdx1* is found throughout the ventral pancreatic domain and in the intestinal bulb (226). *pdx1* knockdown results in impaired endocrine differentiation from ventral progenitors and impaired exocrine pancreas development, indicating that *pdx1* is essential for proper development of the ventral bud (227). However, the dorsal bud is essentially unaffected, which is consistent with the mouse observation that Pdx1 may not be required for early wave endocrine cell differentiation (227). In addition, *ptf1a* is expressed in the ventral progenitors. These *ptf1a*<sup>+</sup> cells co-express other progenitor markers, supporting a state of multipotency in early ventral bud cells (183). Fate mapping of these *ptf1a*<sup>+</sup> cells showed that they give rise to all pancreatic lineages (228). After the ventral bud progenitor domain completes its expansion, the expression of *ptf1a* becomes limited to acinar cells, where it is critical to maintain acinar cell identity. Suppression of Ptf1a activity in differentiated zebrafish pancreatic acinar cells promotes their reprogramming into insulin<sup>+</sup> cells (71). This finding clearly demonstrates the plasticity of pancreas tissues and exemplifies how key transcription factors play crucial roles in maintaining the stability of each cell fate.

Similar to mammals, the bHLH transcription factor Neurod1 is also critical for the induction of endocrine cell fates (229-231). Interestingly, Although *neurod1* is expressed in endocrine progenitors and maintained in all nascent endocrine cell types, knockdown of *neurod1* in zebrafish results in an absence of  $\alpha$  cells and a significant reduction of  $\delta$  cells and ghrelin cells, but no significant change in  $\beta$  cell number (230,231). However, contrary to rodent models, the transcription factor Ngn3 is not necessary for the formation of zebrafish pancreatic endocrine cells. Zebrafish *ngn3* is not expressed in the pancreas, and *ngn3* null mutants do not display any apparent endocrine defects. Rather, the role of *ngn3* is replaced in zebrafish by another bHLH transcription factor, *ascl1b*, which works in concert with *neurod1* to regulate zebrafish endocrine formation (230). Nonetheless, beyond the endocrine initiation steps, the transcriptional program regulating later endocrine subtype specification appears to be conserved between mammals and

zebrafish. For example, the transcription factor Arx plays the same role in zebrafish and mammals: repression of  $\beta$  cell fate and promotion of  $\alpha$  cell fate. Knockdown of *arx* completely abolished  $\alpha$  cell development (232).

Altogether, the conservation of the genetic controls of pancreas differentiation, together with its experimental strengths, has defined zebrafish as a unique and powerful model in which to elucidate the molecular mechanisms of pancreas development. For example, it was first shown in zebrafish studies that differentiation of endocrine cells from pancreatic progenitors requires suppression of Bone morphogenetic protein (BMP) signaling. Subsequently, the usage of BMP inhibitor has been adopted into the protocol for the *in vitro* differentiation of  $\beta$  cells from embryonic stem cells (233-235). Thus, this system has demonstrated its capacity to provide translatable targets for  $\beta$  cell restoration strategies.

### **iii. Pancreatic hormones in zebrafish metabolic regulation**

Zebrafish and mammals exhibit a similar metabolic profile with regards to nutrient usage and storage (216,236). Furthermore, zebrafish share cellular metabolic enzymes as well as signaling pathways that control both glucose and lipid metabolism (237). For example, both Glucagon and GLP-1 can be derived from zebrafish proglucagon peptides, and both Glucagon and GLP-1 have been found to play important roles in zebrafish glucose regulation (238-240). The sequence alignment of these peptides among zebrafish, mouse and human reveals that zebrafish *proglucagon* gene products are strongly conserved (241). As in mammals, zebrafish initiate gluconeogenesis during fasting, and this process is highly regulated by Glucagon (242). Conversely, increased blood glucose dampens Glucagon release (243,244). Moreover, stimulation of the Glucagon receptor increases intracellular cAMP levels and represses the key liver gluconeogenesis gene *phosphoenolpyruvate carboxykinase* (*pepck*), showing that zebrafish share many downstream signaling pathways with mammals (237). Interestingly, GLP-1 in fish does not exhibit incretin effects, but rather potently acts to increase glucose via promoting liver glucogenolysis and gluconeogenesis (242,245). Several studies indicate that in teleost fishes, the Glucagon and GLP-1 signals may share



similar cellular and physiology functions during development and in metabolism control; this is further discussed in Chapter three.

As in mammals, insulin signaling in zebrafish exhibits essential roles in energy storage and glucose metabolism. Injection of insulin into adult zebrafish lowers blood glucose and increase glycogen synthesis (246). Moreover, ablation of  $\beta$  cells in either larvae or adult zebrafish induces hyperglycemia (247,248). These data indicate that insulin secreted from  $\beta$  cells in zebrafish has conserved roles in the regulation of metabolism, and that the loss of  $\beta$  cells or insulin signaling in zebrafish can be used to mimic diabetes. There are two *insulin* genes (*insa* and *insb*) in the zebrafish genome (249). In contrast to observations in mouse, the expression pattern of the two zebrafish genes are very different (249). While *insa* is mainly expressed in pancreatic  $\beta$  cells, *insb* is strongly expressed in the zebrafish head during development. The divergent expression patterns of two genes underlie different functions during development. As gene duplication often results in functional subdivision among orthologues via changes to expression pattern, *insb* may execute the mammalian insulin role of regulating central neuron system development and function (129). Because of this, studies of *insa* gene in zebrafish provide the opportunity to specifically analyze the role of pancreatic insulin pancreas development without confounding influences from the central neuron system. Gene duplication events also affected the insulin receptor (*insr*), resulting in two *insr* genes in zebrafish, *insra* and *insrb*. Both genes exhibit ~68% identity to human *INSR*, and can respond to human insulin stimulation (250). Activation of insulin signaling in zebrafish stimulates conserved downstream signal transduction pathways, such as Akt phosphorylation (250). The specific roles of insulin signaling in zebrafish are further discussed in chapters four and five.

#### **iv. $\beta$ cell regeneration models in zebrafish**

In order to perform  $\beta$  cell ablation and regeneration studies, several approaches have been developed in both embryonic and adult zebrafish. Relative to mammals, zebrafish exhibit a much higher potential for  $\beta$  cell regeneration following injury, which makes zebrafish a more powerful model for investigating which cellular and molecular pathways

are involved, or could be involved, in regeneration. As with mouse models, zebrafish  $\beta$  cell ablation can be induced via chemical and surgical interventions, as well as using transgenic models. It has been shown that a single high dose of STZ can be injected into adult zebrafish and that this results in  $\beta$  cell death and hyperglycemia. Zebrafish recovered rapidly following the STZ treatment: increased proliferation of islet cells and ductal cells was observed shortly after STZ treatment (247) and normal blood glucose was achieved within 2 weeks (247,251). This model was further exploited to analyze how the regeneration of other tissues is influenced by a hyperglycemic environment, in models of diabetic complications (252). Besides STZ-induced  $\beta$  cell ablation, surgical partial pancreatectomy has also been carried out in adult zebrafish (247). Extensive duct cell regeneration and fibrotic tissue were observed following surgery, and extensive  $\beta$  cell proliferation was deduced as the primary mechanism of  $\beta$  cell regeneration in this model.

To date, two zebrafish transgenic models have been reported that permit temporally-controlled  $\beta$  cell ablation and regeneration. In the first, *Tg(ins:cre)Tg(ins:loxp-BFP-loxp-DTA)*, the cell lethal toxin, Diphtheria Toxin-A (DTA), was specifically expressed in  $\beta$  cells after Cre-mediated recombination (224). This method of ablation triggered  $\beta$  cell neogenesis from pancreatic duct progenitors. In the second model, the *Tg(ins:NTR)* model, bacterial *Nitroreductase* (*NTR*) was induced in  $\beta$  cells under control of the insulin promoter. *NTR* activates the innocuous prodrug metrodinazole (MTZ), thereby producing a cytotoxic product that induces cell death. Addition of dissolved MTZ to the media rapidly and thoroughly induces cell death exclusively within *NTR*<sup>+</sup>  $\beta$  cells.  $\beta$  cells rapidly regenerate following the removal of MTZ (253,254). This model has been used throughout this dissertation and discussed extensively in the following chapters.

Interestingly, as in mouse models, compensatory  $\beta$  cell production in response to excessive nutrition is observed in both zebrafish larvae and juveniles (255). Also, over-fed zebrafish developed obesity and exhibited pathophysiology similar to obese humans and mice, which further justifies the investigation of insulin resistance-induced  $\beta$  cell compensation using this zebrafish (256). It is also been shown that adult zebrafish recover from hyperglycemia and impaired glucose metabolism that is induced by

persistent glucose exposure, suggesting the presence of  $\beta$  cell regeneration mechanisms that respond to insulin signaling (257).

Altogether, these studies reveal that the cellular and molecular pathways for pancreatogenesis and  $\beta$  cell regeneration are conserved between zebrafish and mammals. The condensed embryonic developmental period and rapid rate of  $\beta$  cell regeneration make zebrafish uniquely suited for studying these processes, and has contributed to the comprehensive understanding of *in vivo*  $\beta$  cell regeneration processes. Understanding the cellular origins and molecular triggers for  $\beta$  cell regeneration under different environmental conditions will lead to the development of rational drug targets for diabetes treatment. The following chapters will further discuss how these complex biological mechanisms are unified, and how tissue regeneration mirrors development.

## **I.F. Rationale and Research Aims**

Insufficient functional  $\beta$  cell mass underlies the development of diabetes mellitus. Regeneration of new  $\beta$  cells will allow for reestablishment of balanced metabolism and provides a justified foundation hope for a diabetes cure. Both *in vitro* and *in vivo*  $\beta$  cell regeneration approaches recapitulate certain aspects of embryonic pancreas development. As two of the key functional genes in the pancreas, *glucagon* and *insulin* gene products not only play an essential role in regulating metabolism, but also can mediate the balance between  $\alpha$  and  $\beta$  cell quantities during embryonic development. Under normal physiological conditions, glucagon secreted from  $\alpha$  cells and insulin secreted from  $\beta$  cells act as counter-balanced Yin-Yang factors to maintain glucose metabolism; imbalance of these hormones leads to metabolic disease. For example, the *glucagon* gene is strongly activated after  $\beta$  cell injury or impaired insulin signaling (165). The restoration of balance between these two hormones is achievable by increasing the number of  $\beta$  cells or decreasing  $\alpha$  cells (165). The surprising result that spontaneous  $\alpha$  cell to  $\beta$  cell transdifferentiation occurs after extreme  $\beta$  cell ablation (160) indicates that the mature pancreas has innate potential to correct this misbalance. Furthermore, it is likely that the machinery controlling  $\alpha$  and  $\beta$  cell quantities responds directly to insulin and glucagon input and this then help the organism sense and correct the unbalanced hormonal state. In

this study, I have used two approaches to introduce a state of insufficient insulin signaling, and then I analyzed the impact of glucagon/GLP-1 and insulin on  $\beta$  cell generation/regeneration. The overall hypothesis of my dissertation is that:

**Glucagon and Insulin signaling pathways exhibit counter-balanced effects in regulating pancreatic endocrine cell quantities, and that this feedback regulation is crucial for regenerating new  $\beta$  cells following  $\beta$  cell injury or in states of insulin deficiency.**

**To test this hypothesis, I have pursued the following specific aims:**

**Aim 1/ Chapter three:** Characterize the role of Glucagon/GLP-1 in  $\beta$  cell regeneration following severe  $\beta$  cell ablation. In this Aim, I have used the NTR-induced  $\beta$  cell ablation, transgenic zebrafish models, HOT-Cre lineage tracing, as well as genetic and pharmaceutical manipulation to investigate the role of *glucagon* gene products in  $\beta$  cell neogenesis.

**Aim 2/ Chapter four:** Investigate the role of insulin in regulating pancreatic endocrine homeostasis using approaches that deplete insulin signaling but not  $\beta$  cells, *per se*. In this Aim, I have used genetic methods to inhibit the production of the insulin peptide or to block insulin signaling in target cells, to uncover a novel role for insulin signaling in regulating the differentiation of pancreatic progenitor cells.

**Aim 3/ Chapter five:** Examine the impact of insulin in regulating acinar cell fate stability and the development of the exocrine pancreas. In this aim, I have investigated the impact of insulin signaling on exocrine pancreas development, which may give insights into the role of insulin signaling in acinar cell fate maintenance and transformation.

## CHAPTER TWO: Materials and methods

### II.A. Zebrafish maintenance and strains

Zebrafish were raised under standard laboratory conditions at 28.5°C. All animal procedures were conducted in accordance with OLAW guidelines and were approved by the Indiana University Institutional Animal Care and Use Committee.

The following transgenic lines in Table 2-1 were used in the experiments. *Tg(gcga:Cre; cryaa:YFP)<sup>s962</sup>*, *Tg(sst2:Cre; cryaa:YFP)<sup>s963</sup>* and *Tg(hs:loxP-mcherry-STOP-loxP-dnIRS2-GFP)* were constructed and generated by meganuclease transgenesis as described (222). To construct the *Cre* transgenes, *glucagon* promoter (gift of F. Argenton) or *sst2* promoter was subcloned into *ins:Cre; cryaa:Venus*. A 2 kb *sst2* promoter region was amplified from the CH211-232H16 zebrafish genomic clone (CHORI) using the oligos: 5'-GCATG AATTC AGCCT CTATG TCCTT CGTCT and 5'-GCATG GATCC TGCTG CTTCT TTAAC TCAG. To construct the *hsp70l-loxP-mCherry-STOP-loxP-dn-irs2a-GFP* transgene, the N-terminus of *irs2a* was amplified from BAC DKEYP-24D6 using the oligos: 5'- GGCGC GCCAC CATGG CGAGT CCGCC GCCG and 5'-CTCGC CCTTG CTCAC CATGG CTGCC ATGCT GTCAG T and EGFP was amplified from using: 5'-ACTGA CAGCA TGGCA GCCAT GGTGA GCAAG GGCGA G and 5'- CGAGC TGTAC AAGTA AAGCG GCCGC. The two resulting products were fused by PCR and cloned into plasmid *hsp70l-loxP-mCherry-STOP-loxP-H2B-GFP\_cryaa-cerulean* (Addgene #24334) using *AscI* and *NotI*.

For Hot-Cre lineage tracing, heat shock activations were performed at 38.5°C for 20 minutes at 3 dpf. If  $\beta$  cell ablation was required, larvae were recovered at 28.5°C for 3 hours before ablating  $\beta$  cells. In order to induce *dnIRS2-GFP* overexpression via HotCre system, heat shock activations were performed at 38.5°C for 20 minutes at the time point indicated in the legends.

## II.B. Detection of protein, mRNA, and cell proliferation

Whole mount immunofluorescent staining and western blot were used in detecting the protein signal. *In vitro* hybridization and quantitative PCR were used to detect mRNA expression. In order to detect cell proliferation, EdU incorporation assays were performed as described (258).

The following primary antibodies in Table 2-2 were used for immunofluorescent staining. Alexa Fluor-conjugated antibodies were used for visualization (Life Technologies). For western blot, mouse anti p-Akt<sup>473</sup> (cell signaling) has been used. The expression of p-Akt<sup>473</sup> is determined by normalizing to  $\alpha$ -tubulin that is visualized by mouse anti- $\alpha$  tubulin antibody (Santa cruz).

For quantitative PCR, we enriched for endodermal organs by manual dissection of the digestive system with watchmakers forceps (Dumont #5, F.S.T.), which included pancreas, intestine, and liver from 4, 5, and 7 dpf larvae. 20-30 endoderm preparations were pooled for each condition to minimize variability between dissections. mRNA was extracted with Trizol (Life Technologies) and reverse transcribed with iScript (BioRad). The Mastercycler *Realplex* PCR system (Eppendorf) was used with Sybr Green mix (Invitrogen) and Mytaq (BioLine) to generate Ct values. The relative expression of each sample was determined by normalizing to *laminB1* (*lmnb1*) or  $\beta$ -actin using the relative standard curve method (259). The primer sets in Table 2-3 have been used for quantitative PCR analysis.

For *In vitro* hybridization, the following primer sets in Table 2-4 have been used to synthesize the probe. The probe template was PCR amplified from cDNA with the described primer sets and then *in vitro* synthesized and labeled using Dig RNA labeling kit T7 (Roche). The *In vitro* hybridization procedure was then performed as described before (260) and stained with BM purple AP Substrate (Roche). For immunofluorescence *In vitro* hybridization, Vector Red (Vector Inc.) was used.

## II.C. Microinjections

*H2B-RFP* mRNA, *dnIRS2-GFP* mRNA, *insulin* mRNA and *sox32* mRNA was transcribed with SP6 mMessage machine kit (Invitrogen) in vitro. In order to label the differentiated dorsal pancreatic endocrine cells, 100 pg *H2B-RFP* mRNA were injected into zygotes. Anti-dsRed (Clontech) and Alexa568 antibodies (Life Technologies) were used to amplify the signal. In order to block insulin signaling or over expression insulin, 200pg *dnIRS2-GFP* mRNA or 200pg *Insulin* mRNA were injected into zygotes. Anti-GFP (Aves Labs) and Alexa488 antibodies were used to amplify the *dnIRS2-GFP* expression signal. In order to induce endoderm specific differentiation, 200pg *sox32* mRNA was injected into zygotes.

In order to knock specific gene expression, the following antisense morpholinos list in Table2-5 (Gene Tools LLC) were injected into zygotes. Among them, *gcga*MO and *arxa*MO are translation blocking morpholino. The morpholinos were designed to bind at the translation start site of the target mRNA which then block the protein translation. The *insa*MO and *insb*MO are splicing morpholino. The morpholino were designed to bind at the exon-intron junction of the target gene pre-mRNA. Injection of the morpholino will interrupt the pre-mRNA splicing and results in exon deletion or alternative splicing products.

Specificity of morpholino knockdown was addressed in multiple ways: First, significant off-target effects were unlikely in *insulina*, *glucagon* or *arxa* morphants, as we observed no gross morphological defects, which are characteristic of such off-target and other non-specific toxic effects in morphants (261). Secondly, *gcga* and *arxa* morpholino phenotypes recapitulated phenotypes seen in mouse with knockdown of components of the glucagon signaling pathway (262,263) or Arx(85) . Thirdly, regeneration of  $\beta$  cells in the *gcga* knockdown was rescued by injection of recombinant human Glucagon peptide or the Glp1 receptor agonist Exendin-4, but not the Glp1 receptor antagonist Exendin9-39 (Figure 5A-G; S16). Finally, injection of a second *arxa* morpholino (*arxa*MO2 = 5'-ATGTT TGTAT CGTCC TCAGT CGTGC) produced identical phenotype in the islet (R.M.A., unpublished). To address morpholino efficacy, *gcga*MO eliminated glucagon protein in the islet (Figure 4D'), and *arxa*MO specifically eliminated expression of an

*arxa-GFP* DNA reporter construct that was co-injected into zygotes (R.M.A. unpublished).

For detecting *insa*MO and *insb*MO efficiency, mRNA was extracted from 2 dpf control and MO-injected embryos and cDNA was synthesized as described above. The PCR product was then run through 1% agarose gel and image was taken with Carestream Gel Logic system. 5'-CATTCCTCGCCTCTGCTTC and 5'-GGAGAGCATTAAGGCCTGTG primer set were used for detection *insa*MO efficiency and 5'-CAGACTCTGCTCACTCAGGAAA and 5'-GCGTGTAATGGTCATTTATTGC primer set were used for detection *insb*MO efficiency. The alternative splicing products were cut from the gel and sent for sequencing.

#### II.D. Drug/chemical treatments

The chemical used in the experiments were listed in Table 2-6. Specifically, For  $\beta$  cell ablation, *Tg(ins:CFP-NTR)<sup>s892</sup>* or *Tg(ins:Flag-NTR)<sup>s950</sup>* animals were incubated in 0.1% DMSO (Sigma)  $\pm$  10 mM Metronidazole (MTZ, Sigma) in egg water. After ablation (generally 24 hrs), embryos were washed extensively with egg water, and recovered for 1-16 days.

For peptide treatments, a mixture of KCl (0.2M), phenol red (0.1%), and either vehicle, recombinant human Glucagon (Sigma), Exendin-4 (Sigma), Exendin9-39 (Sigma) was injected into the pericardial sac of each embryo following ablation. The peptides were dissolved in H<sub>2</sub>O for stock solution (1mM) and then stored at -20°C. The final working solution is 1 $\mu$ M and 4nl/larvae were injected. The total mass of each drug injected was 0.004 pM/larva. For Glucose/Mannose injection, stock Glucose (Sigma) or Mannose (Sigma) was dissolved in H<sub>2</sub>O and stored in -20°C at the concentration of 10%. The Glucose/Mannose working concentration is 0.25% and 4nl was injected into the pericardial sac of each embryo. Thus, Glucose or Mannose was injected with a final mass of 10 ng/larva. After injection, embryos recovered at 28°C for 5 h before ablation.

For inhibition of Akt pathway, *Tg(ins:CFP-NTR)<sup>s892</sup>* embryos were incubated in 0.1% DMSO  $\pm$  1 $\mu$ M wortmannin (sigma) from 14hpf and fixed at 30 hpf or 42 hpf. For



inhibition of MAPK pathway, *Tg(ins:CFP-NTR)<sup>s892</sup>* embryos were incubated in 0.1% DMSO± 100µM U0126(sigma) from 14 hpf and fixed at 32 hpf.

## **II.E. Glucose measurements**

Glucose measurement in zebrafish embryos was performed as described (248) using a glucose assay kit (Biovision). Briefly, pools of 15-20 embryos were counted and homogenized in glucose measurement buffer (10 ul/ embryo). Three groups were used for each treatment condition. To measure glucose level, glucose oxidase specifically oxidizes free-glucose generating a compound that reacts with the glucose probe to produce resorufin, which can be detected colorimetrically (O.D. 570 nm). Specific measurement steps were performed according to the manufacturer provided instructions. The fluorescence signal was read using Spectra Max-M5 (Molecular device). The data was displayed with the mean glucose amount per larvae.

## **II.F. Transplantation**

In order to analyze the specific roles of Insulin signaling on endoderm, chimeric zebrafish embryos have been generated by cellular transplantation procedure as described by previous research. In order to induce endoderm specific replacement, *sox32*mRNA were synthesized in vitro and injected (200pg/embryo) into the donor embryos at one cell stage. 4ng *sox32*MO was injected into zygotic host embryos to promote the contribution of donor cells. In order to inhibit insulin signaling in the donor cells, 200pg *dnIRS2-GFP* mRNA was injected. *Tg(sox17:GFP)* were used in the donor embryos to mark and trace the differentiated endoderm. Wildtype were used in the host embryos. Both donor and host embryos were dechorionated at blastula stage and then placed in the transplantation agar molds. Transplantation was performed under the dissecting scope and directed by an oil-filled Hamilton syringe with a micrometer drive.

In order to analyze the differentiation of blastula stem cells in developed zebrafish host larvae system, a novel transplantation approach has been used. *Tg(sox17:GFP)* donor embryos were injected with 200pg *sox32*mRNA alone or co-injected with 200pg

*dnIRS2-GFP*mRNA. Dechorinated blastula stage donor embryos were placed in the agar molds and host larvae were mounted in a 35 mm petri dish with 2% methylcellulose. Transplantation was performed with the same system and needles as above. About 20-40 cells were extracted from the donor and injected into 4 dpf host larvae at endoderm cavity behind liver and above pancreas. To create the  $\beta$  cell ablated host environment, *Tg(ins:Flag-NTR;cryaa:mCherry)<sup>s950</sup>* embryos were treated  $\pm$  10 mM MTZ from 3dpf to 4dpf and MTZ was removed 2 h prior transplantation. To determine whether blood vessels can integrate with transplanted donor tissue, *Tg(ubi:zebrabow)* donor embryos with ubiquitous RFP expression were injected with 200pg *sox32*mRNA and transplanted into 4 dpf *Tg(flk1:GFP)* host larvae.

## **II.G. Data and statistical analysis**

For the pancreatic cell counting, the images stacks were taken using confocal microscope (Zeiss, LSM700) with the interval of 1.5  $\mu$ M. The images were then analyzed using imageJ software and target cell populations were counted manually. The data was then displayed as mean cell number/ larvae. Dissecting fluorescence scope (Leica M205) was used for the whole amount imaging for zebrafish embryos.

Data are expressed as mean  $\pm$  SD, and statistical significance was tested by ANOVAs analysis or Student's t-test (as noted in figure legend). For two way ANOVAs analysis, Bonferroni posttest was used for statistical analysis. For one way ANOVAs analysis, Tukey Post Hoc test was used for statistical analysis and statistic was analyzed using Prism software. Significant differences from controls are indicated as follows:  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*).

Table 2-1. Transgenic lines used for experiments.

Name	Usage	Reference
<i>Tg(neurod1:GFP)<sup>nl1</sup></i>	Pan-endo marker <sup>Chapter 3, 4</sup>	Obholzer, N. et al., 2008 (264)
<i>Tg(sox17:GFP)<sup>s870</sup></i>	Endoderm marker <sup>Chapter 3,4</sup>	Sakaguchi, T. et al., 2006 (265)
<i>Tg(ins:Cre; cryaa:YFP)<sup>s924</sup></i>	$\beta$ cell lineage tracing <sup>Chapter 3</sup>	Hesselson, D. et al., 2009 (222)
<i>Tg(Ins:CFP-NTR)<sup>s892</sup></i>	$\beta$ cell ablation <sup>Chapter 3,4</sup>	Curado, S. et al., 2007 (266)
<i>Tg(gcga:GFP)<sup>ia1</sup></i>	$\alpha$ cell marker <sup>Chapter 3,4</sup>	Pauls, S. et al., 2007 (267)
<i>Tg(ins:dsRed)<sup>m1018</sup></i>	$\beta$ cell marker <sup>Chapter4</sup>	Anderson, R. et al., 2009 (258)
<i>Tg(ins:Flag-NTR; cryaa:mCherry)<sup>s950</sup></i>	$\beta$ cell ablation <sup>Chapter3,4</sup>	Andersson, O. et al., 2012 (248)
<i>Tg(hs:loxp-mCherry-STOP-loxp-H2BGFP)<sup>s925</sup></i>	Lineage tracing <sup>Chapter3,4</sup>	Hesselson, D. et al., 2009 (222)
<i>Tg(flk1:GFP)<sup>s844</sup></i>	Blood vessel marker <sup>Chapter3,4</sup>	Jin, S. et al., 2005 (268)
<i>Tg(ubi:zebrabow)<sup>a131</sup></i>	transplantation <sup>Chapter4</sup>	Pan, Y. et al., 2013 (269)
<i>Tg(ela3l:cre; cryaa:YFP)</i>	Acinar cell lineage tracing <sup>Chapter3,4</sup>	Hesselson, D. et al., 2011 (183)
<i>Tg(hs:cre)<sup>VU297</sup></i>	Inducible cre <sup>Chapter4,5</sup>	Ni, T. et al., 2012 (270)
<i>Tg(gcga:Cre; cryaa:YFP)<sup>s962</sup></i>	$\alpha$ cell lineage tracing <sup>Chapter3,4</sup>	Ye, L. et al., 2015 (271)
<i>Tg(sst2:Cre; cryaa:YFP)<sup>s963</sup></i>	$\delta$ cell lineage tracing <sup>Chapter3</sup>	Ye, L. et al., 2015 (271)
<i>Tg(hs:loxp-mcherry-STOP-loxp-dnIRS2-GFP)</i>	Inducible <i>dnIRS2</i> expression <sup>Chapter4,5</sup>	unpublished
<i>Tg(fabp10:dsRed; ela3l:GFP)<sup>gz12</sup></i>	Liver and acinar marker <sup>Chapter5</sup>	Anderson, R. et al., 2009 (258)

Table 2-2. Primary antibodies used for immunofluorescence staining.

<b>Name</b>	<b>Raised species</b>	<b>Dilution</b>	<b>Company</b>
anti-GFP	chick	1:500	Aves Labs
anti-Insulin	guinea pig	1:100	Biomeda
anti-Glucagon	mouse	1:100	Sigma
anti-somatostatin	rabbit	1:100	Serotec
anti-dsRed	rabbit	1:250	Clontech
anti-PCNA	mouse	1:100	Alcam
anti-PHH3	rabbit	1:00	Cell signaling
anti-Alcam	mouse	1:00	Zn-8
anti-cleaved Cap3	rabbit	1:100	Cell signalling
anti-Pdx1	guinea pig	1:100	gift of Dr. C. Wright

Table 2-3. Primers used for qPCR analyses.

Gene name		Strand	Sequence
<i>laminB1</i>	<i>lmnb1</i> <sup>chapter3</sup>	sense	5'-ACCCGCGGCAAGAGAAAGCG
		antisense	5'-TCCTGCCATCGGCTGGTCCT
<i>preproinsulin-a</i>	<i>insa</i> <sup>chapter3</sup>	sense	5'-TCTGCTTCGAGAACAGTGTG
		antisense	5'-GGAGAGCATTAAGGCCTGTG
<i>Preproglucagon-a</i>	<i>gcga</i> <sup>chapter3</sup>	sense	5'-AAGGCGACAGCACAAGCACA
		antisense	5'-GCCCTCTGCATGACGTTTGACA
<i>aristaless related homeobox-a</i>	<i>arxa</i> <sup>chapter3</sup>	sense	5'-AAAAGCAAGTCGCCCACCGT
		antisense	5'-AATTGGGCGGCAGGTGCATGT
<i>mafa</i>	<i>mafa</i> <sup>chapter3</sup>	sense	5'-ATTGTTTCGCCGGGCTGTGTT
		antisense	5'-TGCTTTTGGCACAACCGGCA
<i>mafba</i>	<i>mafba</i> <sup>chapter3</sup>	sense	5'-CGCCAAACTGTGTTTGCGCTGA
		antisense	5'-AGGCGGCTTTAACGGGAGAAGT
<i>neuronal differentiatoin1</i>	<i>neurod1</i> <sup>chapter3</sup>	sense	5'-ACGCAGCGCTGTGATATACCGA
		antisense	5'-TCGCGTTCAACTGGGCGTTCAT
<i>elastase 3 like</i>	<i>ela3l</i> <sup>chapter3</sup>	sense	5'-GCTGAGCCTGTGACACTGAG
		antisense	5'-TCTCTGTGTGTTGGTTTTCTGG
<i>trypsin</i>	<i>trp</i> <sup>chapter3,4,5</sup>	sense	5'-AGACCGTCTCTCTGCCTTCA

		antisense	5' - CAACACGCCATGATAACGAC
<i>isl LIM</i> <i>homeobox 1</i>	<i>isl1</i> <sup>chapter3</sup>	sense	5' - AGCAGCAGCAACCCAACGACAA
		antisense	5' - TGCACCTCCACTTGGTTTGCCT
<i>opsin 1, short-</i> <i>wave-sensitive 1</i>	<i>opn1sw1</i> <sup>chapter3</sup>	sense	5' -CCCAAATGGGCGTTCTACCT
		antisense	5' -CAAGGACCATCCCGTCACAA
<i>insulin receptor-a</i>	<i>insra</i> <sup>chapter4</sup>	sense	5' -CGGCTCGCTGTGTTGTATTG
		antisense	5' -TACTGTCCCTCCTCTCACGG
<i>insulin receptor-b</i>	<i>insrb</i> <sup>chapter4</sup>	sense	5' -TCGCCTACATCTTGTGCCTC
		antisense	5' -AGCTCAAGCCCCTGAAATCC
<i>pancreatic and</i> <i>duodenal</i> <i>homeobox 1</i>	<i>pdx1</i> <sup>chapter4</sup>	sense	5' - ACACGCACGCATGGAAAGGACA
		antisense	5' -GCGGGCGCG AGATGTATTTGTT
<i>β-actin</i>	<i>β-actin</i> <sup>chapter4</sup>	sense	5' - GGCACGAGAGATCTTCACTCCCC
		antisense	5' - GGGGAAAACAGCACGAGGGGC

Table 2-4. Primers used for In Situ Hybridization.

Gene name		Strand	Sequence
<i>preproglucagon-a</i>	<i>gcga</i> <sup>chapter3</sup>	sense	5'- ATAAGCGAGGAGACGATCCA
		antisense	5'- gctaatacgactcactataggGCAATGAA GCCATCAGTTCTC
<i>glucagon receptor-a</i>	<i>gcgra</i> <sup>chapter3</sup>	sense	5'- GAGTGTCAACGCAGTTCAGT
		antisense	5'- gctaatacgactcactataggCTGTCCGT CTGCATCACACT
<i>Glucagon receptor-b</i>	<i>gcgrb</i> <sup>chapter3</sup>	sense	5'-CCGCTCATATTTGTGCTGCC
		antisense	5'- gctaatacgactcactataggAGCGGAGC CTTCATTGTTGA
<i>insulin receptor-a</i>	<i>insra</i> <sup>chapter4,5</sup>	sense	5'-GCTCGTGCGCGTGTTCATAT
		antisense	5'- gctaatacgactcactataggTTTCCGTG GCCTGAGTTC
<i>insulin receptor-b</i>	<i>insrb</i> <sup>chapter4,5</sup>	sense	5'- GGCTGGACACATCTGTGGTTG
		antisense	5'- gctaatacgactcactataggCGGTGGAG GACAATTATATCGTAG
<i>pancreatic and duodenal homeobox 1</i>	<i>pdx1</i> <sup>chapter4</sup>	sense	5'- GGGAGACTGCAGGTAGAGCA
		antisense	5'- gctaatacgactcactataggGCCTTTTG CCAATCTGTTTGC

\*Note: all the antisense primers contain the T7 promoter sequences which indicate as lower case characters.

Table 2-5. Morpholino sequences

<b>Name</b>	<b>Target gene</b>	<b>Morpholino sequence</b>	<b>Dose</b>
standard control MO	no target in <i>Danio rerio</i> <i>chapter3</i>	5'- CCTCT TACCT CAGTT ACAAT TTATA	4 or 8 ng
<i>arxa</i> MO	<i>aristaless related</i> <i>homeobox-a</i> <i>chapter3</i>	5'-TATCG TCGTC GTACT GACTG CTCAT	4 ng
<i>gcga</i> MO	<i>preproglucagon-a</i> <i>chapter3</i>	5'- GGCAA AATAC TGGAC GCCTT TCATT	8 ng
<i>insa</i> MO	<i>preproinsulin-a</i> <i>chapter4,5</i>	5'- CCTCTACTTGACTTTCTTACCCAGA	4 ng
<i>insb</i> MO	<i>preproinsulin-b</i> <i>chapter4</i>	5'- AAGTTGGAGACGTTGCTCACCCAG C	2 ng
<i>sox32</i> MO	<i>sex determining</i> <i>region Y-box 32</i> <i>chapter4</i>	5'- GCATCCGGTCGAGATACATGCTGT T	4 ng



Table 2-6. Drug treatments

<b>Name</b>	<b>Purpose</b>	<b>Dose</b>
Metronidazole	$\beta$ cell ablation <sup>Chapter 3, 4</sup>	10mM
Human-Glucagon	Gcgr activator <sup>Chapter 3</sup>	0.004pM/larva; 14pg/larva
Exendin-4	GLP-1r activator <sup>Chapter 3</sup>	0.004pM/larva; 16.7pg/larva
Exendin-9-39	GLP-1r antagonist <sup>Chapter 3</sup>	0.004pM/larva; 13.5pg/larva
Glucose	Increase blood glucose <sup>Chapter 3,4</sup>	10ng/larva
Mannose	Osmotic control <sup>Chapter4</sup>	10ng/larva
Wortmannin	PI3K inhibitor <sup>Chapter3,4</sup>	0.1 $\mu$ M
U0126	ERK inhibitor <sup>Chapter3,4</sup>	100 $\mu$ M

## CHAPTER THREE: *Glucagon* is essential for $\alpha$ cell transdifferentiation and $\beta$ cell neogenesis

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### III.A. Summary

The inter-conversion of cell lineages via transdifferentiation is an adaptive mode of tissue regeneration, and an appealing therapeutic target. However, its clinical exploitation is contingent upon discovery of contextual regulators of cell fate acquisition and maintenance. In murine models of diabetes, glucagon-secreting  $\alpha$  cells transdifferentiate into insulin-secreting  $\beta$  cells following targeted  $\beta$  cell depletion, regenerating the form and function of the pancreatic islet. However, the molecular triggers of this mode of regeneration are unknown. Here, using lineage-tracing assays in a transgenic zebrafish model of  $\beta$  cell ablation, we demonstrate conserved plasticity of  $\alpha$  cells during islet regeneration. In addition, we show that *glucagon* expression is upregulated after injury. Through gene knockdown and rescue approaches, we also find that peptides derived from the *glucagon* gene are necessary for  $\alpha$  to  $\beta$  cell fate switching. Importantly, while  $\beta$  cell neogenesis was stimulated by glucose,  $\alpha$  to  $\beta$  cell conversion was not, suggesting that transdifferentiation is not mediated by glucagon/GLP-1 control of hepatic glucose production. Overall, this study supports the hypothesis that  $\alpha$  cells are an endogenous reservoir of potential new  $\beta$  cells. It further reveals that *glucagon* plays an important role in maintaining endocrine cell homeostasis through feedback mechanisms that govern cell fate stability.

### III.B. Introduction

Glucagon and insulin, secreted by pancreatic  $\alpha$  ( $\alpha$ ) and  $\beta$  ( $\beta$ ) cells, respectively, exert opposing regulatory actions on glucose metabolism. Glucagon increases circulating glucose levels by stimulating hepatic glycogenolysis and gluconeogenesis. Conversely, insulin decreases glucose levels by eliciting cellular influx and storage of glucose while

dampening hepatic glucose production. In the pancreatic islet,  $\alpha$  and  $\beta$  cells are physically juxtaposed; each cell type receives and responds to secreted signals from the other to modulate secretory activities (i.e. cell function). For instance, insulin represses glucagon expression and release from the  $\alpha$  cell, while glucagon stimulates  $\beta$  cell insulin secretion (272,273). Deranged regulation of insulin and glucagon signaling consequent to  $\beta$  cell dysfunction or destruction causes diabetes mellitus, a devastating disease that afflicts more than 360 million people worldwide (274). With the loss of insulin signaling, unchecked glucagon signaling drives hyperglycemia and thereby the morbid complications associated with diabetes (165). However, the mechanisms by which acute changes in intra-islet hormone signaling affect islet cell composition or islet cell fates are largely unknown.

Restoration of  $\beta$  cell mass is a conceptually proven approach to cure diabetes (18), but its widespread implementation will require a vast supply of functional  $\beta$  cells derived from either exogenous (e.g. stem cells) or endogenous (e.g. facultative progenitors) sources. It is clear from models of  $\beta$  cell regeneration in the mouse that the mode and extent of pancreatic injury influences mechanisms of regeneration, and these models have revealed that new  $\beta$  cells can arise from several endogenous sources. Firstly,  $\beta$  cell regeneration may be mediated by proliferation of surviving  $\beta$  cells (162). Secondly, neogenesis from duct or duct-associated progenitor cells may occur spontaneously, or in response to injury via pancreatic duct ligation (176,177), though contradictory reports indicate that this process may be rare (179,180,275). Thirdly, spontaneous transdifferentiation of  $\beta$  cells from  $\alpha$  or  $\delta$  cells occurs in conditions of extreme  $\beta$  cell loss (160,170). Likewise, with some limitations,  $\alpha$  and  $\beta$  cells can be interconverted through gain or loss of key pancreatic transcription factors, including *Arx*, *Pax4*, and *Pdx1* (65,86,153). Together these studies have revealed plasticity in the endocrine pancreas, and indicate that other pancreatic endocrine cells may be an exploitable source of new, functional  $\beta$  cells. However, the endogenous extracellular cues triggering  $\alpha$  to  $\beta$  cell transdifferentiation are unknown.

Here, using transgenic zebrafish models of  $\beta$  cell ablation, we have investigated cellular and molecular mechanisms of new  $\beta$  cell formation during regeneration. The zebrafish is an attractive and useful model for studying mechanisms of pancreas

formation and disease repair because of its conserved architecture, composition, development, and attributes that permit rapid loss-of-function analyses (221,244,276,277). In addition, the heightened regenerative capacity of the zebrafish facilitates the discovery of novel organ repair mechanisms that may be present, but dormant, in mammals (278). In this manuscript, we use lineage-tracing tools to demonstrate conserved plasticity of  $\alpha$  cells during regeneration in the zebrafish islet. Importantly, and for the first time, we show that *glucagon* gene activation is responsible for this  $\alpha$  cell fate switch; blockade of this signaling pathway via *glucagon* knockdown nearly extinguishes  $\beta$  cell regeneration. Importantly, our data further suggest that transdifferentiation is not solely dependent on the gluconeogenic properties of glucagon. Overall, this study supports the hypothesis that  $\alpha$  cells constitute an endogenous reservoir of new  $\beta$  cells that is pharmacologically exploitable.

### III.C. Results

#### **$\beta$ cell regeneration occurs by neogenesis in zebrafish.**

To investigate the origin of regenerating  $\beta$  cells, we used transgenic models of conditional  $\beta$  cell ablation. In *Tg(ins:CFP-NTR)<sup>s892</sup>* and *Tg(ins:Flag-NTR)<sup>s950</sup>*, nitroreductase converts metronidazole (MTZ) into a toxic compound that rapidly induces  $\beta$  cell apoptosis (266). Treatment of embryos with MTZ from 3 to 4 days post fertilization (dpf) ablated all  $\beta$  cells, and after its removal,  $\beta$  cell mass rapidly recovered at a rate greater than normal larval neogenesis (Figure 3-1A-F). We observed that free glucose levels were elevated in  $\beta$  cell ablated larvae (Figure 3-1G), confirming the functionality of larval  $\beta$  cells. Free glucose levels peaked one day into the recovery period (1 day post ablation, dpa), but importantly, by 8 dpf there was no difference in glucose levels between ablated and control groups. This restoration of sufficient overall  $\beta$  cell function, despite only partial recovery of  $\beta$  cell mass indicates that individual  $\beta$  cells may be hyperfunctional. To determine whether surviving  $\beta$  cells contributed to islet regeneration in our model, we used a genetic lineage tracing approach (222). Specifically, *Tg(insa:Cre;cryaa:YFP)<sup>s924</sup>* (hereafter *ins:Cre*) was crossed with the inducible reporter line *Tg(hsp70l:loxp-mcherry-stop-loxp-H2B-GFP;cryaa:CFP)<sup>s923</sup>*

(hereafter *hs:CSH*) (222). First, as a control, we heat-pulsed *ins:Cre; hs:CSH* larvae at 3 dpf to mark *insulin*<sup>+</sup>  $\beta$  cells with H2B-GFP and found that 41% of  $\beta$  cells were labeled at 4 dpf (Figure 3-7A,B,G). In accord with previous studies (222), we saw no change in the quantity of H2B-GFP<sup>+</sup>  $\beta$  cell between 1 and 2 days after labeling (Figure 3-7A-D,H). Next, we heat-pulsed *ins:Flag-NTR; ins:Cre; hs:CSH* embryos at 3 dpf, shortly before MTZ treatment and found that in 1 dpa regenerating islets, only 2% of all post-ablation *insulin*<sup>+</sup>  $\beta$  cells were labeled (Figure 3-1H-I; Figure 3-7I; *n*=13). This result indicates that pre-existing  $\beta$  cells do not significantly contribute to regeneration in our model.

To further exclude a significant contribution of pre-existing  $\beta$  cells to islet regeneration in our model, we used *Tg(ins:Kaede)*<sup>s949</sup> fish in which  $\beta$  cells were labeled by the green-to-red photo-convertible fluorescent protein Kaede (248). When Kaede was converted to red at 72 hpf, control (unablated) islets were comprised of two populations of  $\beta$  cells at 96 hpf. Most exhibited yellow (green plus red) fluorescence, indicating *ins*<sup>+</sup> cells that existed during labeling, while some  $\beta$  cells exhibited only green fluorescence, indicating that they were generated in the 24 hr period after labeling (Figure 3-7J,K). In regenerating *Tg(ins:CFP-NTR)*<sup>s892</sup> islets, when Kaede was converted at 72 hpf immediately after MTZ treatment the 1 dpa islets contained only unconverted green *ins*<sup>+</sup> cells (Figure 3-7L-M). Together, our *ins:Cre* and *ins:Kaede* data demonstrate that the essentially all  $\beta$  cells are ablated by MTZ treatment in the NTR model, and that islet regeneration occurs through  $\beta$  cell neogenesis.

### **$\beta$ cells transdifferentiate from $\alpha$ cells during regeneration.**

In mice, severe  $\beta$  cell ablation triggers  $\alpha$  to  $\beta$  cell conversion (160,161). We reasoned that if this switch occurred in our model, that intermediate cell phenotypes would be detected as  $\alpha$  cell character gives way to  $\beta$  cell character. To test this hypothesis, we used triple transgenic *Tg(ins:FlagNTR); Tg(gcga:GFP); Tg(ins:dsRed)* zebrafish in which  $\alpha$  and  $\beta$  cells were marked in green and red, respectively. Although no  $\beta$  cells remained after MTZ treatment at 0 dpa, several GFP<sup>+</sup> dsRed<sup>+</sup> double positive cells were detected at 1 and 2 dpa (Figure 3-1J-K, Figure 3-8). Next, to distinguish between  $\alpha$  to  $\beta$  cell transdifferentiation and *de novo* co-expression of glucagon and insulin during

differentiation of  $\beta$  cell progenitors, we used temporally restricted  $\alpha$  cell lineage tracing. *Tg(gcga:Cre; cryaa:YFP)<sup>s962</sup>* (hereafter *gcga:Cre*) fish were crossed with *hs:CSH* and *gcga:cre; hs:CSH* embryos were heat pulsed at 3 dpf and analyzed at 4 dpf. This approach marked 17% of  $Gcg^+$   $\alpha$  cells and 8.5% of  $\beta$  cells with H2B-GFP, but no  $\delta$  cells (Figure 3-1L; Figure 3-9A-E). This may reflect activity of the *glucagon* promoter in some  $\beta$  cells or their progenitors during neogenesis in embryos/larvae and adults (Figure 3-10). Even so, when we heat-pulsed *gcga:cre; hs:CSH; ins:Flag-NTR* embryos at 3 dpf to mark *gcga*<sup>+</sup> cells, then ablated  $\beta$  cells with MTZ treatment from 3-4 dpf,  $Gcg^+$   $\alpha$  cells were the only cells that remained marked by H2B-GFP (Figure 3-1M). At 2 dpa, we observed that approximately 13.1% of newly generated  $\beta$  cells (1.9 cells out of 14.5 cells/islet) were H2B-GFP marked (Figure 3-1N,O). When this is normalized to the efficiency of *gcga:Cre* it suggests that approximately 11  $\beta$  cells (77%) per recovering islet are derived from *gcga:cre* expressing cells. Since all  $\beta$  cells, including those labeled by *gcga:Cre*, are destroyed by MTZ treatment, this observation indicates that new  $\beta$  cells originated from pre-labeled  $\alpha$  cells.

Additionally, it was recently shown that somatostatin<sup>+</sup> delta ( $\delta$ ) cells spontaneously convert into insulin producing cells in young,  $\beta$  cell ablated mice (170). In order to determine if  $\delta$  cells could also switch identity in zebrafish, we used *Tg(sst2:Cre; cryaa:Venus)<sup>s963</sup>* (hereafter *sst2:Cre*) to similarly trace  $\delta$  cells. *sst2:Cre* labeled 45% of  $\delta$  cells and 1.5% of insulin<sup>+</sup> cells in intact islets, and in regenerating islets only 1% of the new insulin<sup>+</sup>  $\beta$  cells were labeled (Figure 3-2A-E). Concordantly, regenerating islets rarely showed co-localization of insulin and somatostatin (Figure 3-11). These data indicate that  $\delta$  cells do not typically share the capacity of  $\alpha$  cells to transdifferentiate to  $\beta$  cells in response to  $\beta$  cell ablation in zebrafish.

### **$\alpha$ to $\beta$ cell transdifferentiation can be marked by label retaining assay in the zebrafish principal islet.**

In the pancreata of both *gcga:GFP* and *gcga:cre; hs:CSH* fish, we consistently observed a small fraction of GFP marked cells outside the islet that did not stain for either insulin or glucagon (Figure 3-12). These may represent immature cells that express the

*glucagon* promoter construct, but are not yet committed to the  $\alpha$  or  $\beta$  cell fate (pro- $\alpha$  cells) (279). Our genetic tools would not distinguish between  $\beta$  cells formed from such pro- $\alpha$  cells or from the conversion of mature  $\alpha$  cells inside the islet. Thus, to further test whether mature hormone expressing  $\alpha$  cells can transdifferentiate to  $\beta$  cells during regeneration, we used a Label Retaining Cell (LRC) assay to mark a discrete population of endocrine cells in the principal islet (222,280). For these experiments, embryos were injected with mRNA encoding Histone H2B-RFP, which is a stable fluorescent protein that is exponentially diluted by cell division (Figure 3-3A). Zebrafish endocrine cells derived from the dorsal pancreatic bud differentiate, cluster into an islet, and become largely quiescent by 24 hpf, and therefore retain H2B-RFP fluorescence (222). Conversely, ventral pancreatic bud-derived progenitor cells undergo intensive proliferation before differentiating and thus do not retain the fluorescent signal (H2B-RFP<sup>-</sup>) (2). Therefore using this approach makes it possible to discern the population of dorsal pancreatic bud derived endocrine cells as H2B-RFP<sup>+</sup> cells (Figure 3-3B).

We used multiple criteria to demonstrate that the label retaining cells in the principal islet were committed endocrine cells. First, we determined that nearly all H2B-RFP<sup>+</sup> cells in the islet were positive for *neurod*:GFP (a pan-endocrine marker) and *sox17*:GFP (an endoderm marker) and expressed insulin, glucagon, or somatostatin (Figure 3-3C,D, 3-13A-C). A small number of H2B-RFP<sup>+</sup> cells in the principal islet were *kdrl*:GFP positive and this fraction was similar to the size of the *sox17* negative and *neurod*-negative fractions (Figure 3-13D,E), suggesting that the non-endocrine H2B-RFP<sup>+</sup> cells are endothelial cells. The quantity of H2B-RFP<sup>+</sup>  $\alpha$ ,  $\beta$ , and  $\delta$  cells remained constant during development (Figure 3-3E), which indicates that dorsal bud derived  $\alpha$  and  $\delta$  cells are also largely quiescent, as previously reported for  $\beta$  cells (222). Taken together, our data show that the LRC assay marks differentiated, quiescent pancreatic endocrine cells in the principal islet. Moreover, H2B-RFP<sup>-</sup> ventral bud-derived pancreatic progenitor cells continuously differentiate into endocrine cells and contribute to principal islet formation in zebrafish larvae (Figure 3-3E), in accord with previous findings (222,280).

Next, we used the LRC assay to investigate the origin of new  $\beta$  cells in  $\beta$  cell-ablated *Tg(ins:CFP-NTR)<sup>s892</sup>* larvae. We found that 35.7% of new  $\beta$  cells in 1 dpa regenerating islets were H2B-RFP<sup>+</sup>, indicating that they arose from pre-labeled dorsal bud-derived

endocrine cells (Figure 3F,G, red arrowhead). Given our data above that  $\delta$  cells and surviving  $\beta$  cells do not contribute to  $\beta$  cell regeneration, these H2B-RFP<sup>+</sup>  $\beta$  cells most likely arise from H2B-RFP<sup>+</sup>  $\alpha$  cells. In further support of this assertion, a subset of H2B-RFP<sup>+</sup>  $\beta$  cells was also glucagon positive (Figure 3-13F, white arrow). Finally, we examined the expression of Pdx1 in putative transdifferentiating  $\alpha$  cells. Initially Pdx1 is expressed in all pancreatic progenitors, and later in differentiated  $\beta$  and  $\delta$  cells (281). Importantly, the role of Pdx1 as a critical transcription factor for  $\beta$  cell development and maturation is conserved in zebrafish (227,282). In the 4 dpf pancreas, Pdx1 was expressed in  $\beta$  and duct cells, but not in glucagon<sup>+</sup>  $\alpha$  cells (Figure 3-3H). However, after  $\beta$  cells were ablated, many Pdx1<sup>+</sup> Gcg<sup>+</sup>  $\alpha$  cells were detected in the islet (Figure 3-3I,J), consistent with previous observation in mouse models that Pdx1 is expressed in  $\alpha$  cells during  $\alpha$  to  $\beta$  cell transdifferentiation (160,161).

Since pre-existing  $\alpha$  cells switched to  $\beta$  cells in our ablation model, we hypothesized that  $\alpha$  cell mass would decrease unless compensated by proliferation or neogenesis. We found no difference in  $\alpha$  cell mass at any of the stages examined through 20 dpf (Figure 3-14A). To measure proliferation rate, we injected 1 dpa larvae with EdU and found that  $\alpha$  cell labeling was significantly increased in the regenerating group (Figure 3-3K, Figure 3-14B, C). We observed similar results using the M-phase marker phospho-histone H3 (Figure 3-14D-I). No significant  $\beta$  cell proliferation was detected at 1 dpa (Figure 3-S8J-L). This further supports our results above that neogenesis, rather than proliferation of pre-existing  $\beta$  cells, is the primary source of during the initial stage of  $\beta$  cell regeneration in our model. Subsequently, however,  $\beta$  cell proliferation may also contribute to the restoration of  $\beta$  cell mass in the later stages of regeneration.

Next, we excluded the possibility that any replication of dorsal bud derivatives confounded the LRC assay by titrating the injected *H2B-RFP* mRNA. We found that RFP fluorescence was completely lost only with a 16-fold dilution, suggesting that labeled cells can divide three times ( $1 \div (2)^3$ ) and remain detectable (Figure 3-15A-E). Since we found no significant change of the H2B-RFP<sup>+</sup> islet cell number between 0 dpa and 1 dpa (Figure 3-15F-H), and since we only detected  $\leq 2$  EdU<sup>+</sup> H2B-RFP<sup>+</sup> cells per regenerating islet (Figure 3-15I), these data indicate that dilution of H2B-RFP signal by proliferation is negligible. Moreover among the proliferating  $\alpha$  cells, most were found to



be H2B-RFP<sup>-</sup> (Figure 3-15J). Altogether, our data suggest that dorsal bud derived endocrine cells do not proliferate, but that H2B-RFP<sup>+</sup>  $\alpha$  cells directly convert to  $\beta$  cells. The increased rate of H2B-RFP<sup>-</sup>  $\alpha$  cell proliferation may compensate for the loss of  $\alpha$  cell mass due to  $\alpha$  to  $\beta$  cell conversion during regeneration phase. In sum, our data show that  $\beta$  cells formed during islet regeneration arise both from  $\alpha$  cell transdifferentiation (H2B-RFP<sup>+</sup>) and from differentiation of ventral bud-derived pancreatic cells (H2B-RFP<sup>-</sup>), which could be naïve progenitors or ventral bud derived endocrine cells. These findings prompt the question of how these two cellular regeneration sources are coordinately regulated.

### **$\alpha$ cells and *glucagon* are required for $\beta$ cell regeneration.**

To test the necessity of  $\alpha$  cells in islet regeneration, we eliminated them by zygotic injection of *arxa* morpholino (*arxa*MO). The homeobox transcriptional repressor Arx is conserved between humans and zebrafish, and orthologues have 68% identity in their peptide sequences. Importantly, loss of *Arx* results in depletion of  $\alpha$  cells from the endocrine pancreas in human, mouse and fish (85,88,232,283). In *arxa*MO-injected, but not control MO-injected islets, glucagon protein and mRNA were undetectable, though neither morpholino generated general morphological defects (Figure 3-3L, M, Figure 3-S10A-C and data not shown).  $\beta$  cell number was statistically unaffected, and  $\delta$  cell number increased (Figure 3-16D,E). Next, we tested how the loss of Arx influenced  $\beta$  cell regeneration from dorsal and ventral bud-derived sources by co-injecting *arxa*MO and *H2B-RFP* mRNA into *Tg(ins:CFP-NTR)* embryos. In *arxa*MO-injected larvae, the number of H2B-RFP<sup>+</sup> regenerated  $\beta$  cells was significantly reduced compared with control morpholino injected larvae (Figure 3-3N, O), showing that Arx is required for  $\beta$  cell transdifferentiation from  $\alpha$  cells. Interestingly, regenerating islets in *arxa*MO-injected larvae had an increased number of insulin<sup>+</sup> somatostatin<sup>+</sup> cells, suggesting that  $\alpha$  cell loss may facilitate  $\delta$  to  $\beta$  cell transdifferentiation (Figure 3-16F-H). Surprisingly, loss of Arxa also resulted in a dramatic decrease of H2B-RFP<sup>-</sup>  $\beta$  cell regeneration (Figure 3-3N, O). Thus, in addition to our earlier findings, which show that  $\alpha$  cells can be direct

precursors of  $\beta$  cells,  $\alpha$  cells may also provide local or systemic cues that regulate neogenesis from ventral bud-derived precursors.

To test whether glucagon could be such a cue, we isolated endodermal organs from ablated and control embryos (Figure 3-4A, Figure 3-17), then used quantitative PCR to examine *insulin* (*insa*) and *glucagon* (*gcga*) expression (other genes we examined are listed in Supplemental Table 3-1). As expected, *insulin* was depleted at 0 dpa, but showed recovery at 1 and 3 dpa (Figure 3-4B). In contrast, *gcga* expression mirrored free glucose measurements: elevated at 0 and 1 dpa, but returned to baseline by 3 dpa (Figure 3-1G, Figure 3-4B). *In situ* hybridization at 4 and 5 dpf showed that *gcga* expression was restricted to the pancreas and that expression in control and regenerating islets followed the same trend, which qualitatively supports the qPCR data (Figure 3-18A-H). Zygotic injection of translation-blocking *glucagon* morpholino (*gcga*MO) into *Tg(gcga:GFP)* zygotes caused no gross developmental defects (Figure 3-18I); however, it eliminated glucagon protein expression, increased *gcga:GFP*<sup>+</sup> cell number, and decreased  $\beta$  cell formation (Figure 3-4C-E). Moreover, using the LRC assay we found that *gcga*MO impaired ventral, but not dorsal bud-derived  $\beta$  cell formation (Figure 3-4F; Figure 3-19). Conversely, the expansion of  $\alpha$  cell mass was associated with increased  $\alpha$  cell proliferation and neogenesis from duct associated progenitors (Figure 3-20), consistent with the endocrine phenotypes reported in mutant mice deficient in the glucagon pathway (139,141,284,285).

We next asked if  $\beta$  cell regeneration was impacted following *glucagon* knockdown by co-injecting *gcga*MO and *H2B-RFP* mRNA into *Tg(ins:CFP-NTR)* zygotes, and found that  $\beta$  cell regeneration from both *H2B-RFP*<sup>+</sup> and *H2B-RFP*<sup>-</sup> sources was significantly reduced (Figure 3-5A,D,G). The effect of *gcga*MO on  $\beta$  cell regeneration and  $\alpha$  cell transdifferentiation was further confirmed by *gcga:cre; hs:CSH*  $\alpha$  cell lineage tracing. We found a significant reduction of total  $\beta$  cells and *H2B-GFP*<sup>+</sup>  $\beta$  cells in *gcga*MO-injected regenerating islets (Figure 3-5H-J). These results mirror our *arxa*MO results in which  $\alpha$  cell loss influences  $\beta$  cell regeneration from both cellular sources, and is consistent with both local and systemic functions of glucagon.

### **Glucagon or Exendin-4 infusion restores $\beta$ cell regeneration.**

Selective proteolysis results in production of several peptide products from the *glucagon* gene, including glucagon and Glucagon-Like Peptide-1 (GLP-1), which signal through distinct receptors. Using *glucagon* gene knockdown, we could not distinguish between actions of these peptides on  $\beta$  cell regeneration. Because human glucagon binds and activates zebrafish Glucagon receptor (*Gcgra*), and the GLP-1 receptor agonist Exendin 4 (Ex-4) activates zebrafish GLP-1 receptor (*Gcgrb*) (245,286), we therefore parsed their roles by infusing recombinant human glucagon and Ex-4 into the circulation of  $\beta$  cell-ablated zebrafish larvae. When we infused glucagon or Ex-4 into control  $\beta$  cell-ablated embryos, we observed that glucagon increased H2B-RFP<sup>-</sup>  $\beta$  cell production, but had no significant effect on transdifferentiating H2B-RFP<sup>+</sup> cells (Figure 3-5A-C,K). Neither Ex-4 nor the GLP-1 receptor antagonist Ex-9-39 significantly affected  $\beta$  cell regeneration (Figure 3-21). In contrast, in *gcga*MO-injected  $\beta$  cell-ablated embryos we found that either glucagon or Ex-4 infusion rescued  $\beta$  cell regeneration from both H2B-RFP<sup>+</sup>  $\alpha$  cells and H2B-RFP<sup>-</sup> ventral bud-derived sources (Figure 3-5A,D-G; Figure 3-22). Together, these data suggest that glucagon and GLP-1 may have permissive effects on  $\alpha$  to  $\beta$  cell transdifferentiation. In addition, both pathways can modulate  $\beta$  cell neogenesis from the ventral bud-derived sources, which are likely duct-derived progenitors. In accord with these interpretations, we found that the glucagon receptor (*gcgra*) and the GLP1 receptor (*gcgrb*) were expressed widely in zebrafish endodermal organs at 4 and 5 dpf, including the pancreas and liver (Figure 3-23).

A primary action of glucagon signaling is stimulation of hepatic glucose production. Previous studies showed that glucose is critical for  $\beta$  cell differentiation (287) and is a potent  $\beta$  cell mitogen (288-290). Thus, we performed glucose infusions to determine whether glucagon might influence  $\beta$  cell production indirectly through liver-derived glucose. Injected glucose was cleared from larvae by 6 hours, and free glucose was decreased in *gcga*MO-injected larvae (Figure 3-24). In concordance with a previous report (255), glucose treatment modestly increased  $\beta$  cell mass (Figure 3-6A-C). Similarly, when we infused regenerating larvae with glucose, we observed an increased number  $\beta$  cells (Figure 3-6D-F). Quantification of label retaining populations showed that the increased regeneration was confined to the ventral bud derived H2B-RFP<sup>-</sup>

progenitors, which indicated that glucose did not directly affect  $\alpha$  cell transdifferentiation (Figure 3-6F). In support of this interpretation, we found no increase in expression of Pdx1 in  $\alpha$  cells after treatment with glucose (Figure 3-25). Finally, to further dissect the roles of hyperglycemia and glucagon signaling on regeneration from each  $\beta$  cell source, we tested whether glucose could rescue  $\beta$  cell regeneration in *gcga* morphants. While we observed an increase in regenerating  $\beta$  cells in both glucose and glucagon treated larvae, only glucagon could restore H2B-RFP<sup>+</sup>  $\beta$  cell formation (Figure 3-6G-K). Together, our data support a model in which *glucagon per se* is necessary for  $\alpha$  to  $\beta$  cell conversion, while glucose levels regulated by glucagon or GLP-1 signaling drive  $\beta$  cell neogenesis from ventral bud progenitors (Figure 3-6L).

### III.D. Discussion

Diabetes results from the depletion or dysfunction of  $\beta$  cells, and their replacement will be central to a cure. Even though  $\beta$  cell replacement strategies can be effective in principle, they are limited by  $\beta$  cell availability (54), necessitating the identification of novel exogenous or endogenous sources of therapeutic  $\beta$  cells. Here, we have exploited a zebrafish model of conditional  $\beta$  cell ablation, in which functional  $\beta$  cell mass rapidly recovers, to study mechanisms of vertebrate  $\beta$  cell regeneration. We have investigated the origins of regenerating pancreatic  $\beta$  cells in this zebrafish  $\beta$  cell ablation model using two approaches: a cre-lox based genetic lineage tracing approach that indelibly marks endocrine cells with temporal precision and a non-genetic cell tracing approach that marks quiescent, differentiated endocrine cells (222). Using these approaches, we distinguished two sources of new  $\beta$  cells: a ventral pancreatic bud-derived source, which may include “naïve” duct-associated progenitor cells that have been previously described (2,291), and non- $\beta$  endocrine cells ( $\alpha$  cells in particular) that transdifferentiate into  $\beta$  cells. Thus, we have shown for the first time the spontaneous, facultative conversion of  $\alpha$  cells into  $\beta$  cells in an organism other than mouse. Most importantly, we identified that activation of *glucagon* is required for  $\beta$  cell regeneration from both precursor pools. Furthermore, our results demonstrate that key physiological actions of *glucagon* in regulating blood glucose are conserved with mammals, just as with insulin

(221,244,248); in particular, we found that in zebrafish glucagon activity is correlated with blood glucose levels, and that *glucagon* expression is up-regulated in the absence of  $\beta$  cells. These findings further validate the zebrafish as a model for studying human metabolic disease.

In our study, we have found that  $\alpha$  to  $\beta$  cell transdifferentiation was strongly diminished in *glucagon* knockdown embryos and that this process could be restored by infusion of either glucagon or Exendin-4. Thus, we have uncovered a novel mechanism that regulates the spontaneous  $\alpha$  to  $\beta$  cell transdifferentiation through the action of physiologically relevant peptide hormone signaling pathways; in particular, peptides derived from the *glucagon* gene. However, since exogenous treatment with glucagon/Exendin-4 did not further increase  $\alpha$  to  $\beta$  transdifferentiation, these results suggest that *glucagon* gene products are important permissive signals facilitating the destabilization of  $\alpha$  cell identity. Moreover, our data suggest that activation of the *glucagon* gene may coordinately regulate  $\beta$  cell regeneration from  $\alpha$  cells and pancreatic duct-associated progenitor cells. The effect of *glucagon* on the latter source may act indirectly through increased gluconeogenesis, as glucose potently increased H2B-RFP<sup>+</sup>  $\beta$  cells, even in *gcga* knockdown islets. Interestingly, we found that newly formed H2B-RFP<sup>+</sup>  $\beta$  cells in the principal islet often form contacts with extra-islet H2B-RFP<sup>+</sup>  $\beta$  cells via long cellular extensions. This observation suggests that  $\alpha$  cell transdifferentiation within the islet may help recruit new  $\beta$  cells to the core of the islet, and thus regenerate normal islet structure (L.Y. and R.M.A. unpublished result).

The *glucagon* gene gives rise to multiple active endocrine peptides through selective proteolysis of Preproglucagon by prohormone convertases (PC). The  $\alpha$  cells yield glucagon via PC2 activity, while GLP-1 is generated mainly in intestinal L cells by PC1/3 activity (292). In mammals, glucagon and GLP-1 have counter-regulatory actions on blood glucose: glucagon stimulates hepatic glucose production, while GLP-1 inhibits it through stimulation of  $\beta$  cell and inhibition of  $\alpha$  cell functions (293). However, in our zebrafish ablation model, the regeneration defect observed in *glucagon* morphants could be rescued by injection of either recombinant human glucagon, or the GLP-1 receptor agonist Exendin-4. Indeed, glucagon and GLP-1 have similar effects upon cAMP generation and hepatic glucose production in teleost fish (294). This may be due to loss

of the ancestral GLP-1 receptor, together with the evolved capacity of a duplicated glucagon receptor to bind GLP-1 (242). Thus, we speculate that in fish, the glucagon signaling pathway executes some of the functions of GLP-1 in mammals. In mammalian animal models of diabetes and in human diabetic patients, up-regulation of *Pc1/3* expression and GLP-1 production in  $\alpha$  cells was observed together with increased Glucagon secretion (295-297). Furthermore, GLP-1 production in  $\alpha$  cells is associated with  $\beta$  cell compensation and regeneration in response to  $\beta$  cell injury (298-300). Thus, it is possible that the increased glucagon/GLP-1 signaling observed in our zebrafish  $\beta$  cell ablation model reflects a conserved function of GLP-1 signaling in mammalian  $\beta$  cell regeneration. GLP-1 receptor is abundantly expressed in pancreatic ducts,  $\beta$  cells, and embryonic  $\alpha$  cells (123). While it is unclear whether adult  $\alpha$  cells express GLP-1 receptors, these receptors are expressed in insulin/glucagon dual hormone positive cells in the rat pancreas (122,123). Thus, we anticipate that GLP-1 receptor expression increases in  $\alpha$  cells during transdifferentiation; this will be an area of future study. In addition to the demonstrated functions of GLP-1 signaling during  $\beta$  cell regeneration, we cannot exclude the role of glucagon signaling in this process given the fact that glucagon plays a role in the maturation and function of  $\beta$  cells. For instance, injection of glucagon into chick embryos increases  $\beta$  cell mass and decreases  $\alpha$  cell mass (240). Furthermore, insulin expression is delayed and ultimately reduced in the islets of *Gcgr*<sup>-/-</sup> mice (139,140), and fetal mice treated with *glucagon* morpholinos showed impaired differentiation of early  $\beta$  cells (141). Indeed, we found that both glucagon receptor (*gcgra*) and GLP-1 receptor (*gcgrb*) were expressed throughout the pancreas in zebrafish larvae. Thus, further research is needed to delineate how different peptides derived from the *glucagon* gene work together to restore islet cell composition and function following loss of  $\beta$  cell number.

Due to the limitations of our current tools, we cannot discern whether ventral bud-derived H2B-RFP<sup>+</sup>  $\alpha$  cells also transdifferentiated into  $\beta$  cells, and if these precursors are mature or immature  $\alpha$  cells.  $\beta$  cell neogenesis may involve a glucagon-positive intermediate that may initially exhibit some characteristics of  $\alpha$  cells, but subsequently mature into  $\beta$  cells. Intriguingly, the presence of *insulin*:mCherry and *glucagon*:GFP dual positive cells in the non-ablated larval and adult zebrafish pancreas suggest that this

process may be active in the homeostasis of the islet cell mass. Indeed, it has been proposed that a pro- $\alpha$  cell population exists as a facultative progenitor that exhibits features of  $\beta$  and  $\alpha$  cells in mammals (279), although this proposition contradicts earlier lineage tracing studies (54) that showed that mature  $\beta$  and  $\alpha$  cells arise from distinct lineages. As ~20-65% of  $\alpha$  cells are not marked by the *Glucagon:Cre* transgenic mice (54,79,301,302), it is possible that relevant subpopulations of  $\alpha$  cells have gone undetected in these studies. The development of novel mouse and zebrafish transgenic strains where cre is more faithfully regulated by the endogenous *Glucagon* locus may shed light on this possibility.

Consistent with the hypothesis that  $\alpha$  cells are a relevant source of new  $\beta$  cells in regenerating islets, the loss of  $\alpha$  cells due to the knockdown of *arxa* strongly diminished the regeneration of  $\beta$  cell mass. In *arx*MO-injected islets, we expect that  $\alpha$  cell progenitors are diverted to  $\delta$  and  $\beta$  cell identities, as in the mouse *Arx* knockout, where increases of 200% and 31%, respectively, were observed (85). In *arxa*MO-injected islets, we found that  $\delta$  cell numbers were indeed increased, as was the number of  $\beta$  cells, though the latter was not statistically significant (Figure S10D,E). In regenerating islets, the knockdown of *gcga* phenocopies the loss of  $\alpha$  cells observed in the *arxa* knockdown, providing strong evidence that peptides derived from the *glucagon* gene are critical regulators of  $\alpha$  cell conversion. As in murine disruptions of the glucagon signaling pathway (139,140,164,284), we found that  $\alpha$  cell mass was not only maintained, but expanded through proliferation and neogenesis. That the number of potential  $\alpha$  cell progenitors of  $\beta$  cells was expanded, yet  $\beta$  cell regeneration was depressed further underscores the importance of *glucagon* for cell fate switching. Finally, as hypoglycemia is expected to result from  $\alpha$  cell/*glucagon* depletion, we hypothesize that the observed reduction of  $\beta$  cell neogenesis from ventral pancreatic bud-derived tissues in *arxa* morphants results from a combination of at least two factors: (1) loss of glucose, which acts as a stimulator of  $\beta$  cell differentiation (270), and (2) fewer  $\alpha$  cells acting as progenitors of  $\beta$  cells.

Regeneration studies in mice have shown that the mode and extent of  $\beta$  cell regeneration is dependent on the injury model. Proliferation of surviving  $\beta$  cells predominates after targeted ablation of 70-80% of  $\beta$  cells (22,162), while conversion of  $\alpha$

or  $\delta$  cells to  $\beta$  cells occurs only with extreme  $\beta$  cell loss (>99%) (160,170). Likewise, we would hypothesize that lower levels of  $\beta$  cell ablation in zebrafish may mitigate transdifferentiation in this model. Additionally, plasticity of  $\alpha$  and  $\delta$  cells is not constant with age. In prepubescent mice,  $\delta$  cells, but not  $\alpha$  cells, are competent to transform into  $\beta$  cells after injury (170). In contrast, fetal  $\alpha$  cells can be reprogrammed to  $\beta$  cells by misexpression of *Pdx1*, while  $\alpha$  cells of newborn mice cannot (65). Intriguingly, regenerating islets in STZ-treated adult zebrafish show increased PCNA staining in the mantle region (247), which is consistent with an induced proliferation of  $\alpha$  cells, just as we observe in regenerating larvae (Fig 3K). Whether the endocrine plasticity of the zebrafish larval pancreas is maintained in juveniles, and adults, and whether this is dependent on extreme  $\beta$  cell loss, are important questions that are being addressed in our ongoing studies.

Our data do not distinguish between the possibilities that peptides derived from the *glucagon* gene act directly on  $\alpha$  cells, or that they act indirectly through other tissues (such as the liver) to produce non-glucose intermediate messengers that feed back to  $\alpha$  cells. Indeed, it has been recently shown that hyperplasia of  $\alpha$  cells in *Gcgr* knockouts can be phenocopied by tissue-specific knockdown of the glucagon receptor in hepatocytes (164), revealing that a circulating factor derived from the liver may partially mediate this hyperplastic effect. Thus, it will be important to investigate how  $\alpha$  and  $\beta$  cell mass are affected in this system when challenged by  $\beta$  cell depletion and hyperglycemia. In addition, given the multiple paracrine actions of glucagon in the islet, it will be important to examine the role of the glucagon receptor as well as the GLP-1 receptor specifically within the  $\alpha$  cells, using tissue-specific knockdown or hyperactivation approaches.

In summary, our data are consistent with a model whereby the glucagon/GLP-1 signaling pathway works through two routes to elicit new  $\beta$  cell formation: (1) non-autonomously through stimulation of hepatic glucose production, which can stimulate  $\beta$  cell formation from naïve duct associated progenitor cells (224,287); (2) autonomously within the  $\alpha$  cell compartment to regulate  $\alpha$  cell mass and cell identity (Figure 6L). *Arx* is a potent reprogramming factor that is essential for  $\alpha$  cell development and maintenance (85,86,154). As such, suppression of *Arx* in  $\alpha$  cells will be essential for their conversion



to  $\beta$  cells. Therefore we hypothesize that *arxa* expression is negatively regulated by excessive glucagon/GLP-1 signaling in  $\alpha$  cells. Loss of glucagon/GLP-1 signaling by multiple approaches results in hyperplasia of  $\alpha$  cells and increased glucagon content concomitant with increased expression of *Arx* (165,284). Our data provide a connection between glucagon/GLP-1 signaling and the maintenance of  $\alpha$  cell fate. As  $\alpha$  cells appear to be epigenetically programmed to enable their conversion to  $\beta$  cells in humans (303), investigating how glucagon/GLP-1 signaling pathways link to epigenetic regulators will be an exciting area for further study. Understanding how such extrinsic signals can regulate  $\alpha$  cell stability will facilitate novel diabetes therapies that promote  $\beta$  cell replenishment. Furthermore, together with the observation that  $\alpha$  cells appear to have a heightened capacity for regeneration by neogenesis from duct-associated progenitors (153), our results support the hypothesis that the  $\alpha$  cell pool constitutes an extensive reserve of new  $\beta$  cells. Our data indicate that peptides derived from the *glucagon* gene are factors critical for regulation of this endogenous repair mechanism, and suggest that stimulation of endocrine transdifferentiation may unlock unlimited sources of new  $\beta$  cells.

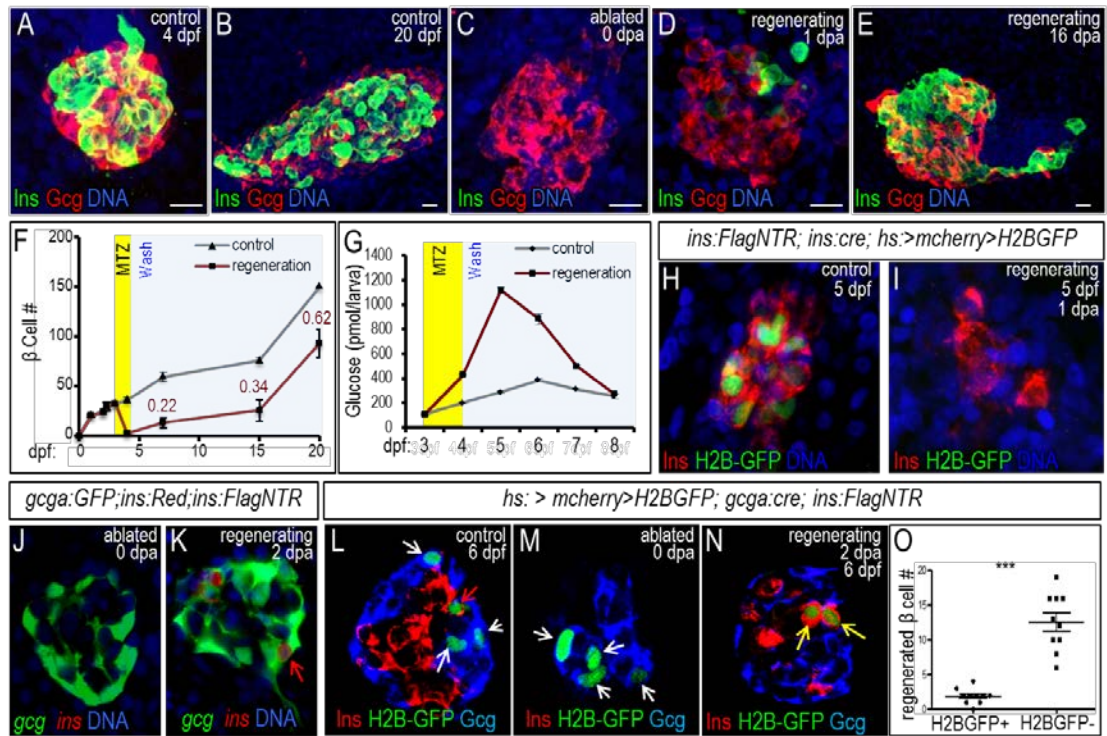


Figure 3-1.  $\beta$  cell neogenesis from  $\alpha$  cell transdifferentiation in zebrafish. (A-E) Confocal projections showing  $\alpha$  (red) and  $\beta$  (green) cells in the principal islet of intact (A,B) and ablated (C,D,E) *Tg(ins:CFP-NTR)* larvae at 0, 1, and 16 days post ablation (dpa). (F) Quantification of *insulin*<sup>+</sup> cells in intact (gray line) and regenerating islets (red line) from 0 to 20 dpf ( $n \geq 3$  for all groups except control 20 dpf,  $n=1$ ). The ratio of  $\beta$  cells in regenerating vs. control islets is indicated. (G) Blood glucose measurements of non-ablated larvae (gray), and larvae in which  $\beta$  cells were ablated from 3-4 dpf (red) ( $n=3$ ). (H,I) Confocal projections of  $\beta$  cell lineage marked islets in 5 dpf control and 1 dpa *Tg(ins:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* larvae.  $\beta$  cells were labeled by inducible H2B-GFP at 3 dpf before ablation and stained for GFP (green) and insulin (red). (J,K) Confocal planes of ablated (J) and regenerating (K) islets in *Tg(ins:Flag-NTR); Tg(gcga:GFP); Tg(ins:Red)* larvae. Red arrows in K indicates *gcga:GFP*<sup>+</sup>*ins:Red*<sup>+</sup>  $\beta$  cells in regenerating islet. (L-N) confocal planes of *Tg(gcga:cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* islets labeled by H2B-GFP before ablation, and stained for GFP (green), insulin (red), and glucagon (blue).  $\alpha$  cells are indicated by white arrows and  $\beta$  cells by red arrows. (L) 6 dpf non-ablated islet, (M) 4 dpf ablated islet at 0 dpa, and (N) 6 dpf islet at 2 dpa. H2B-GFP<sup>+</sup> labeled regenerating  $\beta$  cells are indicated in yellow. (O) Quantification of H2B-GFP<sup>+</sup> and H2B-GFP<sup>-</sup>  $\beta$  cells in 2 dpa islets ( $n=10$ ). Statistical analysis: Student's t-test was used in O.

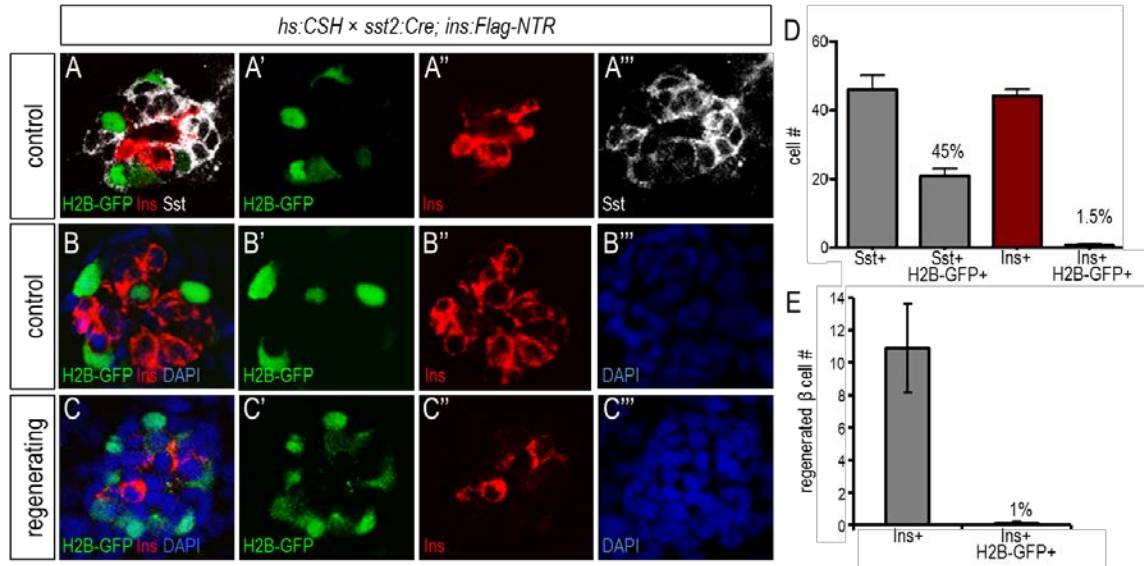


Figure 3-2.  $\delta$  cells but not regenerating  $\beta$  cells are lineage marked by *sst2:Cre*. (A-A''') Merged and single channel images of 5 dpf *Tg(sst2:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* islets that were heat shocked at 3 dpf and stained for GFP(green), insulin (red) and somatostatin (white). Somatostatin<sup>+</sup> $\delta$  Cells are labeled by H2B-GFP. (B, C) Merged and single channel images of 5 dpf non-ablated (B) or 1 dpa (C) *Tg(sst2:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* islets that were heat shocked at 3 dpf and stained for GFP(green), insulin (red) and DNA (blue). (D) Quantification of total somatostatin<sup>+</sup>, somatostatin<sup>+</sup>/H2B-GFP<sup>+</sup>, insulin<sup>+</sup>, and insulin<sup>+</sup>/H2B-GFP<sup>+</sup> cells in non-ablated 5 dpf *Tg(sst2:Cre); Tg(hs:CSH); Tg(ins:Flag-NTR)* islets. 45% of somatostatin<sup>+</sup> cells and 1.5% of insulin<sup>+</sup> cells are marked by *sst2: Cre* ( $n=15$ ). (E) Quantification of insulin<sup>+</sup> and insulin<sup>+</sup>/H2B-GFP<sup>+</sup> cells in 5 dpf regenerating islets. 1% of insulin<sup>+</sup> cells are marked by *sst2: Cre* ( $n=9$ ).

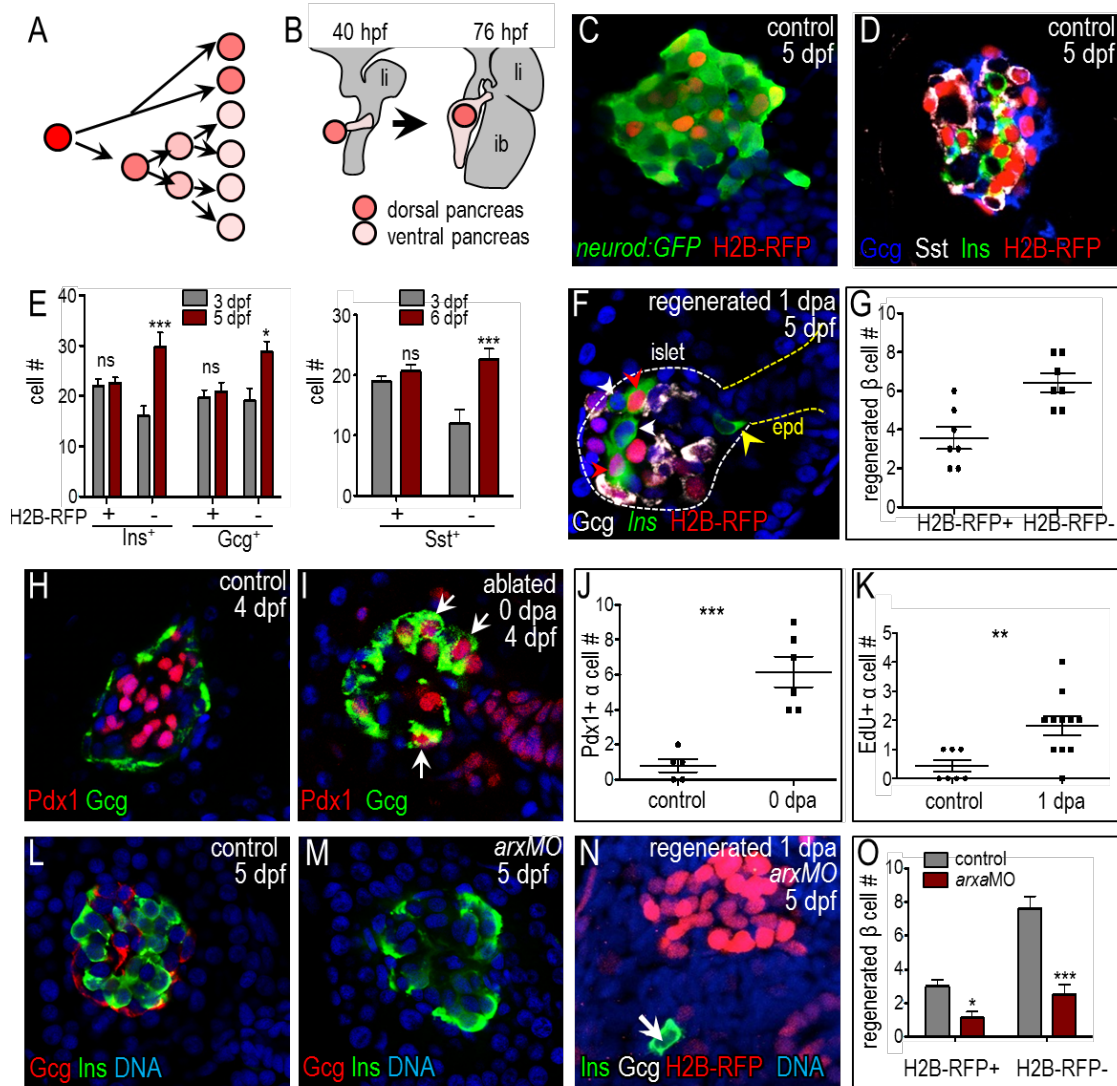


Figure 3-3.  $\alpha$  to  $\beta$  cell transdifferentiation can be marked by label retaining assay in the zebrafish principal islet. (A-B) Schematic of the label retaining assay. (A) Zygotes were injected with *H2B-RFP* mRNA. Fluorescent signal from H2B-RFP protein was diluted by mitosis. (B) The label retaining assay marks quiescent, early differentiated pancreatic endocrine cells derived from the dorsal bud. (C,D) Confocal planes of H2B-RFP-labeled 5 dpf islets. (C) *Tg(neurod:GFP)* ( $n=4$ ), (D) wild type islet stained for insulin (green), glucagon (blue) and somatostatin (white) ( $n=5$ ). (E) Quantification of H2B-RFP<sup>+</sup> and H2B-RFP<sup>-</sup> cells expressing insulin, glucagon, and somatostatin ( $n \geq 3$  for all). (F) H2B-RFP labeled regenerating islet of 5 dpf/1 dpa *Tg(ins:CFP-NTR)* larva stained for CFP (green) and Glucagon (white). Red arrowheads indicate H2B-RFP<sup>+</sup> regenerating  $\beta$  cells, white arrowhead indicates H2B-RFP<sup>-</sup> regenerating  $\beta$  cells in the islet and yellow arrowhead indicates H2B-RFP<sup>-</sup>  $\beta$  cells in the extra-pancreatic-duct region (epd). (G) Quantification of 1 dpa H2B-RFP<sup>+</sup> and H2B-RFP<sup>-</sup>  $\beta$  cells ( $n=7$ ). (H,I) Confocal planes showing Pdx1 (red), glucagon (white) and insulin (green) expression in 4 dpf non-ablated (H) and ablated (I) islets. Pdx1<sup>+</sup>  $\alpha$  cells are marked by white arrows. (J) Quantification of  $\alpha$  cells expressing Pdx1 in 4 dpf non-ablated ( $n=5$ ) and ablated islets ( $n=6$ ). (K) Quantification of proliferating (EdU<sup>+</sup>)  $\alpha$  cells in 5 dpf non-ablated and 1 dpa islets ( $n \geq 7$  islets examined). (L,M) Confocal planes showing glucagon (red) and insulin (green) staining in control MO (L) and *arxa*MO-injected (M) 5 dpf larvae. Glucagon<sup>+</sup> cells are absent from *arxa*MO islets. (N) Confocal projection of *arxa*MO-injected 5 dpf/1 dpa *Tg(ins:CFP-NTR)* islet labeled with H2B-RFP lacking most  $\beta$  cell regeneration. (O) Quantification of 1 dpa  $\beta$  cells in control MO (gray,  $n=16$ ) and *arxa*MO-injected (red,  $n=19$ ) islets. Statistical analysis: Student's t-test was used in J,K and two way ANOVA followed by Bonferroni posttest was used in E,O.

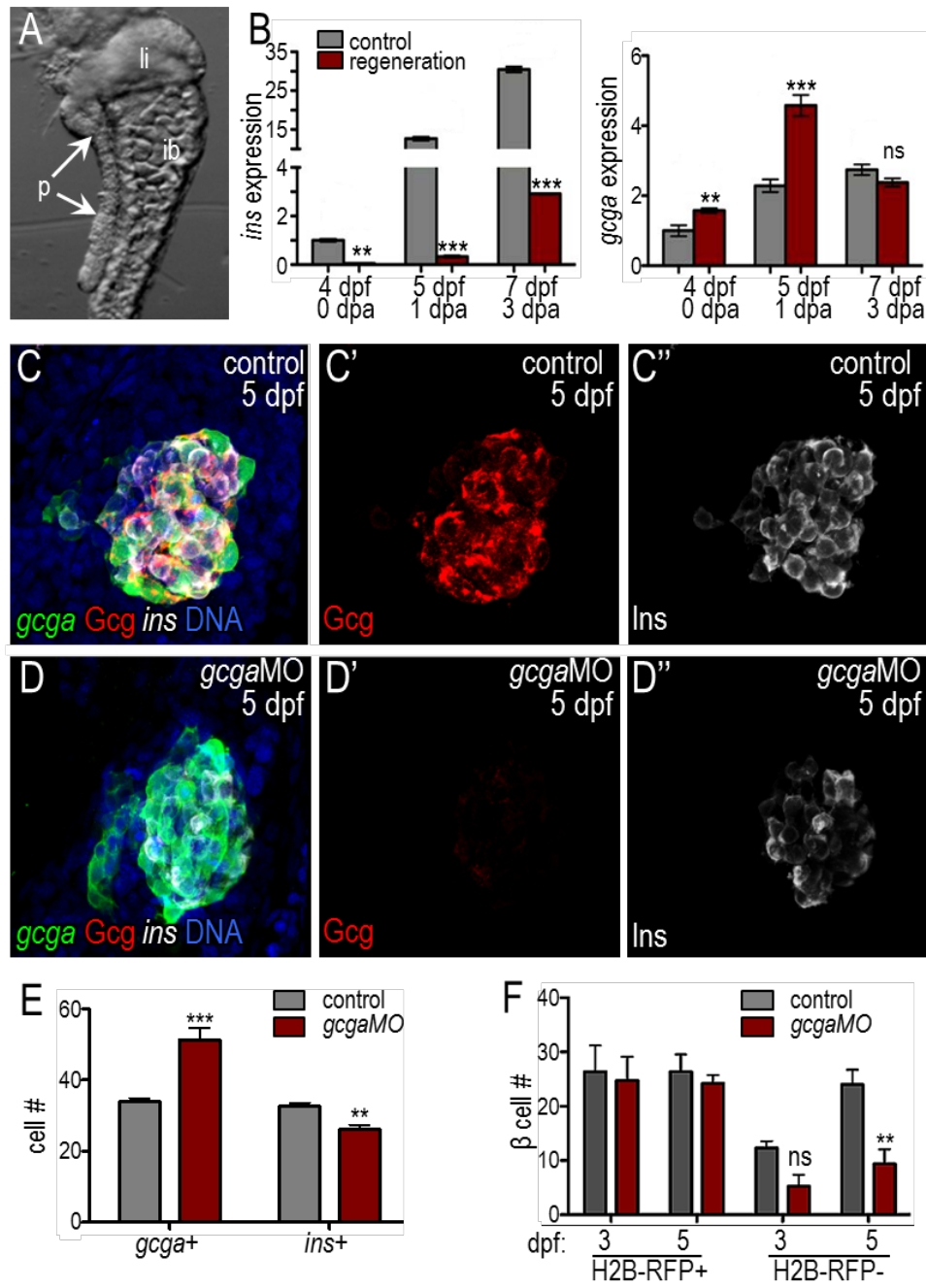


Figure 3-4. *glucagon* is required for islet development. (A) Image of endodermal organs isolated from 4 dpf larvae. p:pancreas, li:liver, ib:intestinal bulb. (B) Quantitative PCR of *insulin* and *glucagon* in control and ablated larvae. *insulin* expression was diminished and *gcga* expression transiently increased during regeneration;  $n=3$  independent experiments. (C-E) *gcgaMO* blocks glucagon protein expression and increases  $\alpha$  versus  $\beta$  cell ratio. (C,D) Confocal projections of 5 dpf control MO (C) and *gcgaMO*-injected (D) *Tg(gcga:GFP);Tg(ins:dsRed)* larvae stained for Glucagon (red). (E) Quantification of *gcga:GFP*<sup>+</sup>  $\alpha$  cells and *insulin*<sup>+</sup>  $\beta$  cells in control MO ( $n=15$ ) and *gcgaMO*-injected ( $n=10$ ) islets. (F) Quantification of H2B-RFP<sup>+</sup> and H2B-RFP<sup>-</sup>  $\beta$  cells at 3 and 5 dpf in control ( $n\geq 3$ ) and *gcgaMO*-injected ( $n=5$ ) islets. Ventral bud derived H2B-RFP<sup>-</sup>  $\beta$  cells are specifically diminished in *gcga* morphants. Two way ANOVA followed by Bonferroni posttest was used in B, E and F for statistical analysis.



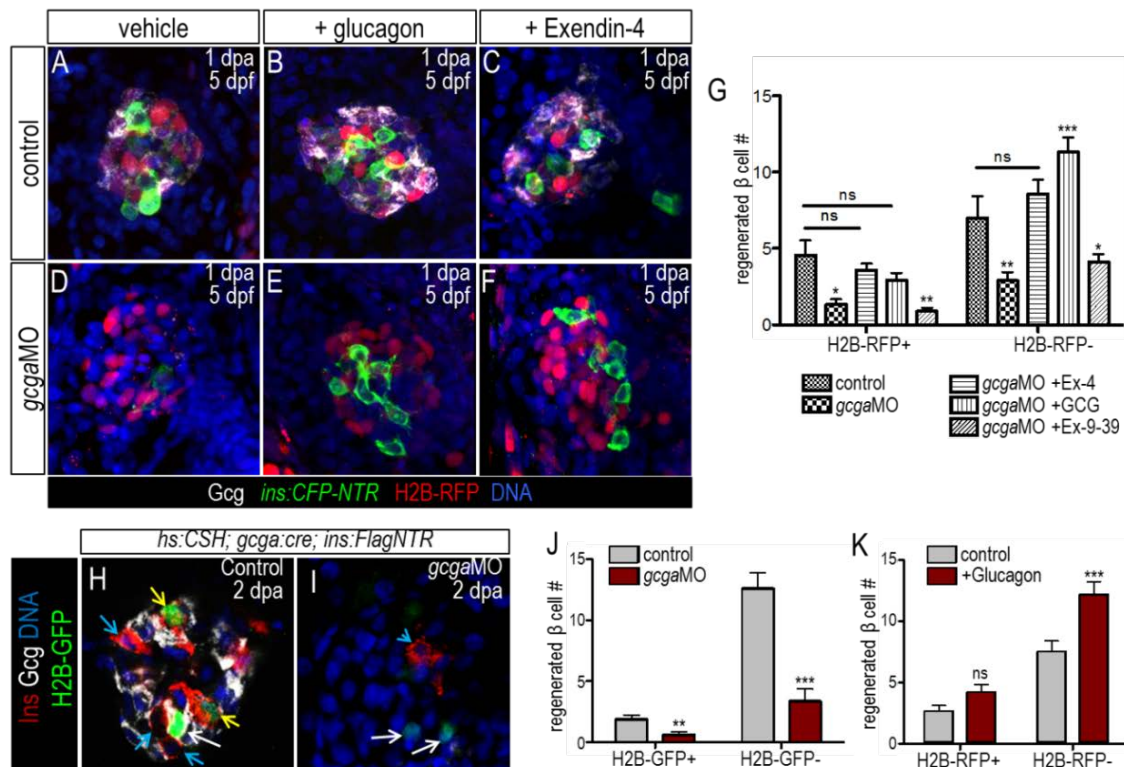


Figure 3-5. Differential regulation of  $\beta$  cell progenitor pools by *glucagon* gene products. (A-F) Confocal projections of 1 dpa H2B-RFP-labeled *Tg(ins:CFP-NTR)* control islets (A-C) and *gcga*MO-injected islets (D-F), and that were treated with vehicle (A,D), recombinant glucagon (B,E), or Exendin-4 (C,F). Islets were immunostained for CFP (green) and glucagon (white).  $\beta$  cell regeneration was restored with glucagon or Ex-4 injection, but not with Ex-9-39 injections. (G) Quantification of H2B-RFP<sup>+</sup> and H2B-RFP<sup>-</sup>  $\beta$  cell number in control regenerating ( $n=7$ ) and *gcga*MO-injected regenerating islets treated with vehicle ( $n=9$ ), glucagon ( $n=13$ ), Ex-4 ( $n=12$ ), or Ex-9-39 ( $n=17$ ). (H-I) Confocal planes of 1 dpa *Tg(gcga:cre);Tg(hs:CSH);Tg(ins:FlagNTR)* control and *gcga*MO injected regenerating islets stained with glucagon (white) and insulin (red).  $\alpha$  cells were labeled (white arrow) at 3 dpf by H2B-GFP before MTZ treatment. Yellow arrow indicates H2B-GFP<sup>+</sup>  $\beta$  cells and blue arrow indicates H2B-GFP<sup>-</sup>  $\beta$  cells. (J) Quantification of H2B-GFP<sup>+</sup> and H2B-GFP<sup>-</sup>  $\beta$  cells in regenerating control ( $n=10$ ) and *gcga*MO-injected islets ( $n=13$ ). (K) Quantification of H2B-RFP<sup>+</sup> and H2B-RFP<sup>-</sup>  $\beta$  cells in regenerating islets treated with vehicle or glucagon ( $n=14$ ). Two way ANOVA followed by Bonferroni posttest (G) and Student's t-test (J, K) and were used for statistical analysis.

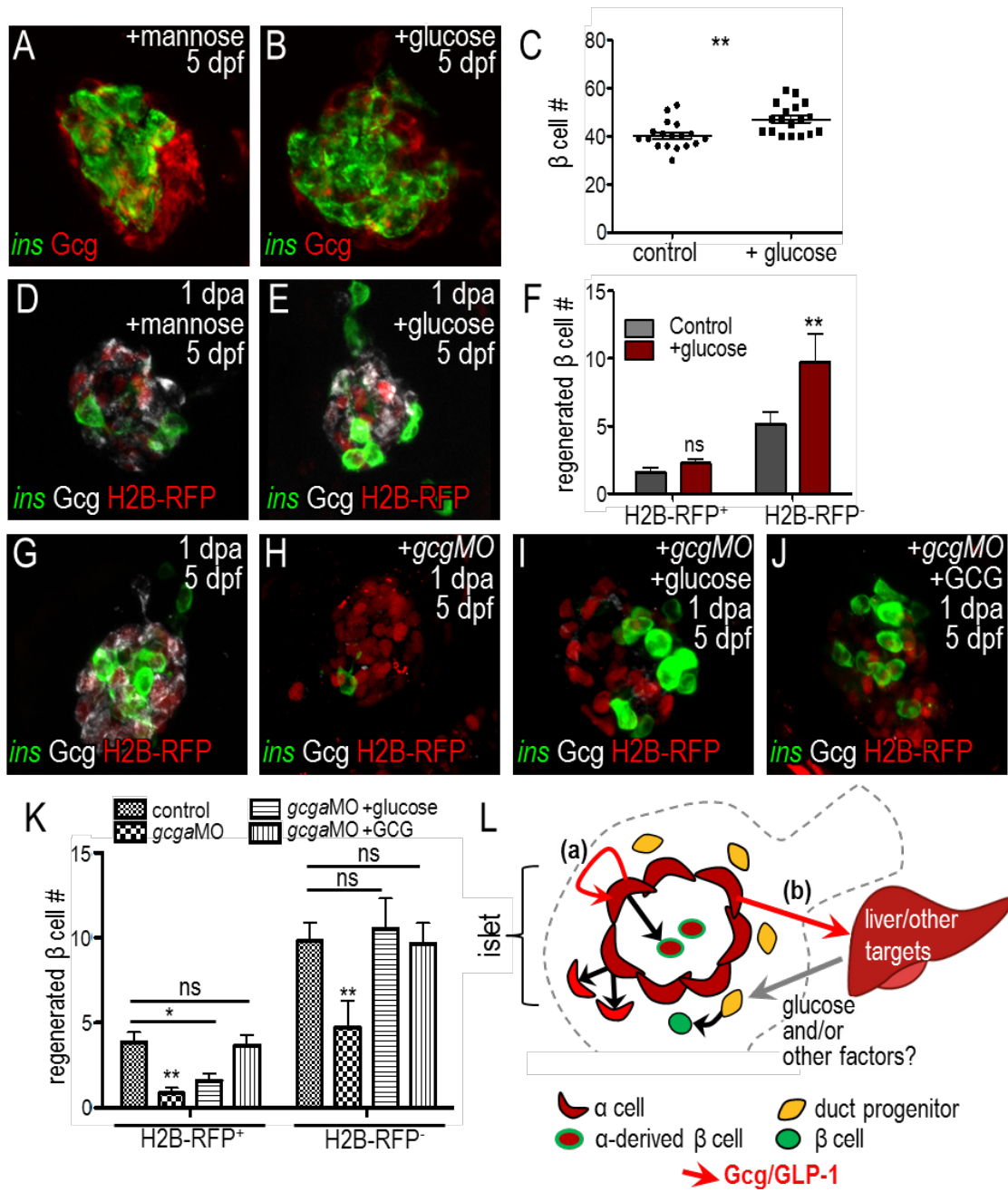


Figure 3-6. Differential regulation of  $\beta$  cell progenitor pools by glucagon and glucose. (A,B) Confocal projections of *Tg(ins:CFP-NTR)* islets with 1 day treatment of mannose (osmolality control, A) and glucose (B) and stained for CFP (green) and glucagon (red). (C) Quantification of *insulin*<sup>+</sup> cells showed that glucose treatment increases  $\beta$  cell mass ( $n=18$ ). (D,E) Confocal projections of 5 dpf/1 dpa *H2B-RFP* mRNA-injected islets that were mannose (D) or glucose-treated (E) during regeneration and stained for CFP (green) and glucagon (white). (F) Quantification of *H2B-RFP*<sup>+</sup> and *H2B-RFP*<sup>-</sup>  $\beta$  cells in control ( $n=7$ ) and glucose treated ( $n=7$ ) islets shows a specific increase in *H2B-RFP*<sup>-</sup> population. (G-J) Confocal projections of 5 dpf/1 dpa *Tg(ins:CFP-NTR)* islets injected with *H2B-RFP* mRNA alone (G), or co-injected with *gcgaMO* (H-J). Samples treated with vehicle (G, H), glucose (I) or with glucagon (J). (K) Quantification of *H2B-RFP*<sup>+</sup> and *H2B-RFP*<sup>-</sup> cells from islets in control ( $n=27$ ), *gcgaMO* ( $n=15$ ), *gcgaMO* +glucose ( $n=17$ ), *gcgaMO* +glucagon ( $n=22$ ). Only glucagon rescued *H2B-RFP*<sup>+</sup>  $\beta$  cell regeneration. (L) A model integrating distinct roles of *glucagon* on  $\beta$  cell formation. Loss of  $\beta$  cells triggers  $\alpha$  cell proliferation and activation of *glucagon*. Increased output of glucagon and GLP-1 drives regeneration from two sources. In the first (a), glucagon/GLP-1 acts autonomously in  $\alpha$  cells to permit their transdifferentiation to  $\beta$  cells. In the second (b), glucagon/GLP-1 acts non-autonomously through intermediates (e.g. liver derived glucose) to drive  $\beta$  cell formation from duct-associated progenitors. Student's t-test (C, F) and One way ANOVA followed by Tukey's Post Hoc test (K) were used for statistical analysis.

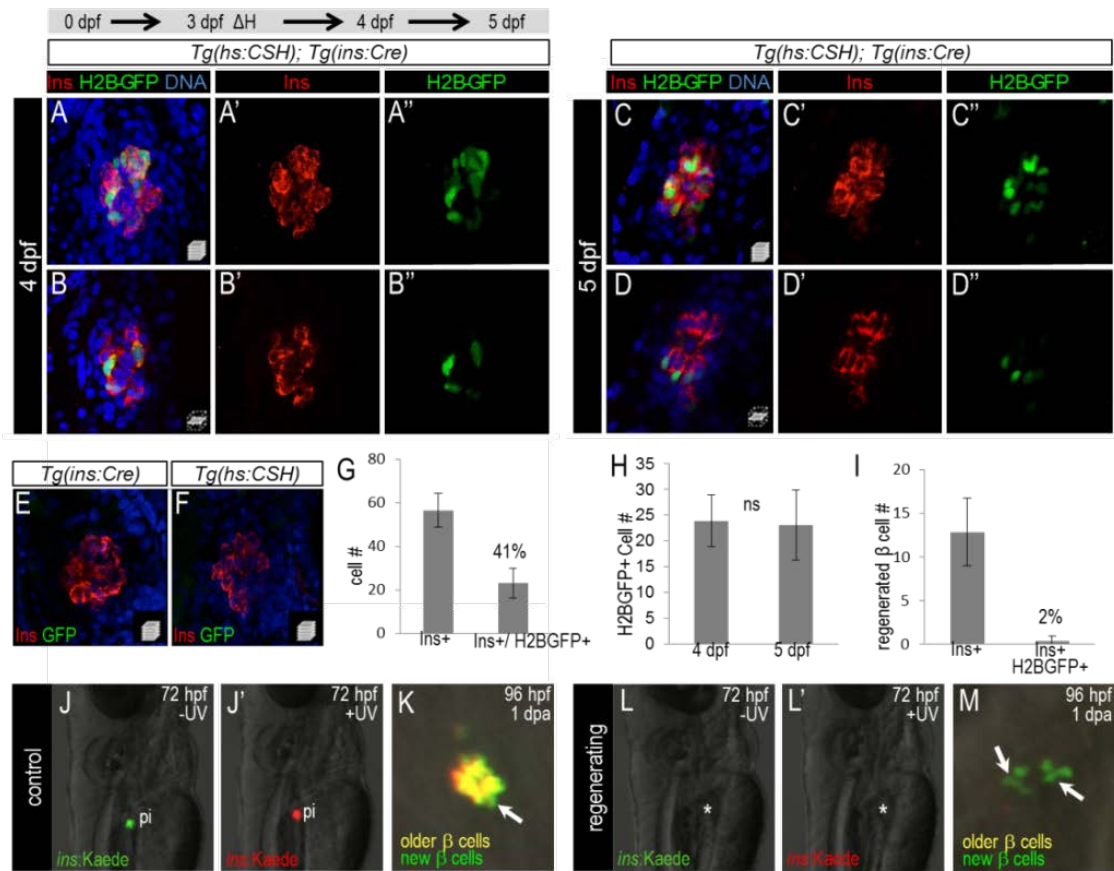


Figure 3-7. Pre-existing  $\beta$  cells do not contribute to  $\beta$  cell regeneration. (A-D) Confocal projections (A,C) and confocal planes (B,D) of *Tg(hs:CSH); Tg(ins:cre)* islets at 4 dpf (A,B) and 5 dpf (C,D) that were heat shocked at 3 dpf and stained for insulin (red), GFP (green), and DNA (blue). Insulin<sup>+</sup> cells were specifically labeled by H2B-GFP. (E, F) Confocal projections of 4 dpf *Tg(ins:cre)* (E) and *Tg(hs:CSH)* (F) islets showed no aberrant or leaky H2B-GFP fluorescence after heatshock. (G) Quantification of insulin<sup>+</sup> and insulin<sup>+</sup>/H2B-GFP<sup>+</sup> cells at 4 dpf showed 41% of  $\beta$  cells were marked ( $n=11$ ). (H) The number of labeled  $\beta$  cells remained constant from 4 to 5 dpf ( $n=7$ ). (I) Quantification of total and H2B-GFP<sup>+</sup>  $\beta$  cells in *the* regenerating islets represented in Fig 1I. Only ~2% of new  $\beta$  cells were labeled ( $n=13$ ). (J-M) Epifluorescent images of *ins:Kaede; ins:CFP* larvae. (J) Green Kaede and (J') converted red Kaede in the  $\beta$  cells of a 72 hpf larva. (K) A 96 hpf larva 24 hours after photo-conversion exhibited  $\beta$  cells present during UV exposure (yellow) as well as new  $\beta$  cells formed after the exposure (green only, arrow). (L) Neither green Kaede (L) nor converted red Kaede (L') are evident at 72 hpf after MTZ-ablation of  $\beta$  cells. (M) A 96 hpf photo-converted larvae 24 hours after photoconversion and cessation of MTZ treatment shows only new  $\beta$  cells (green, arrows). Student's t-test was used in H for statistical analysis.

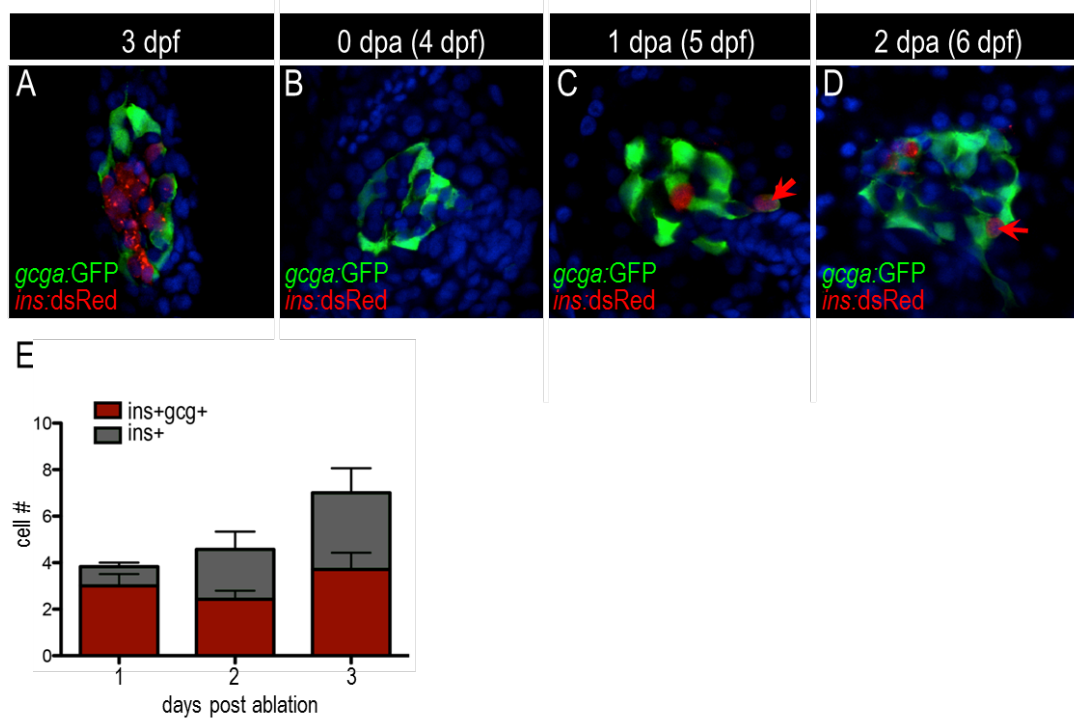


Figure 3-8. Emergence of *gcga:GFP*<sup>+</sup> *ins:dsRed*<sup>+</sup> dual hormone cells during regeneration. (A-D) Confocal planes of *Tg(gcga:GFP)*; *Tg(ins:dsRed)*; *Tg(ins:Flag-NTR)* islets in non-ablated 3 dpf (A), ablated 4 dpf (B), 1 dpa (C), and 2 dpa (D) larvae stained for DNA (blue). *gcga:GFP*<sup>+</sup> *ins:dsRed*<sup>+</sup> dual hormone expressing cells are marked by red arrows. (E) Quantification of *ins:dsRed*<sup>+</sup> single positive and *ins:dsRed*<sup>+</sup> *gcga:GFP*<sup>+</sup> dual positive cells in regenerating islets from 1 to 3 dpa (n≥3).

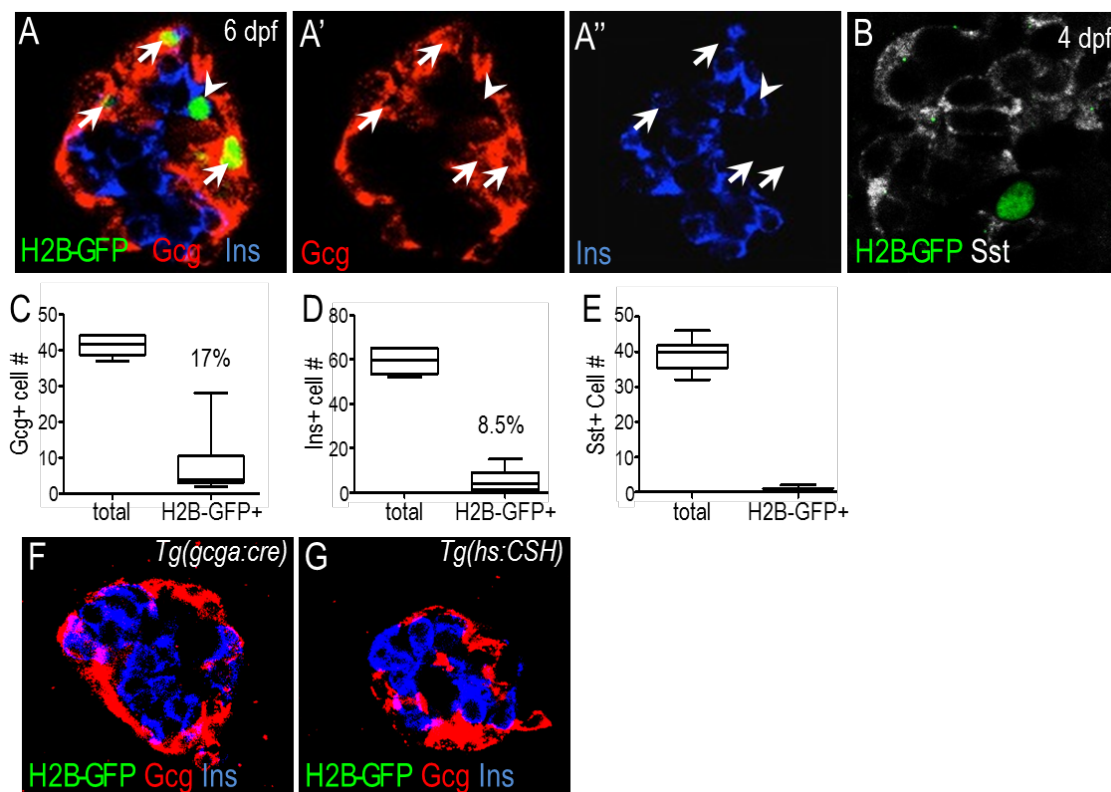


Figure 3-9. Characterization of *glucagon* promoter activity in *Tg(gcga:Cre)*. (A) Merged and single channel confocal planes of 6 dpf *Tg(gcga:cre);Tg(hs:CSH)* islets heat shocked at 3 dpf and stained for GFP (green), Insulin (blue) and glucagon (red). (B) Confocal plane of 4 dpf *Tg(gcga:cre);Tg(hs:CSH)* islet heat shocked at 3 dpf and stained for Somatostatin (white) and GFP (green). (C) Quantification of total glucagon<sup>+</sup> and H2B-GFP<sup>+</sup> glucagon<sup>+</sup> cells in A shows that ~17% of  $\alpha$  cells were marked ( $n=10$ ). (D) Quantification of total Ins<sup>+</sup> and H2B-GFP<sup>+</sup> Ins<sup>+</sup> cells in A shows that ~8.5% of  $\beta$  cells were marked ( $n=6$ ). (E) Quantification of Sst+ and Sst+ H2B-GFP<sup>+</sup> cells in B ( $n=11$ ). (F-G) Confocal planes of 4 dpf *Tg(gcga:cre)* (F) and *Tg(hs:CSH)* (G) islets showed no aberrant or leaky H2B-GFP expression after heat shock.



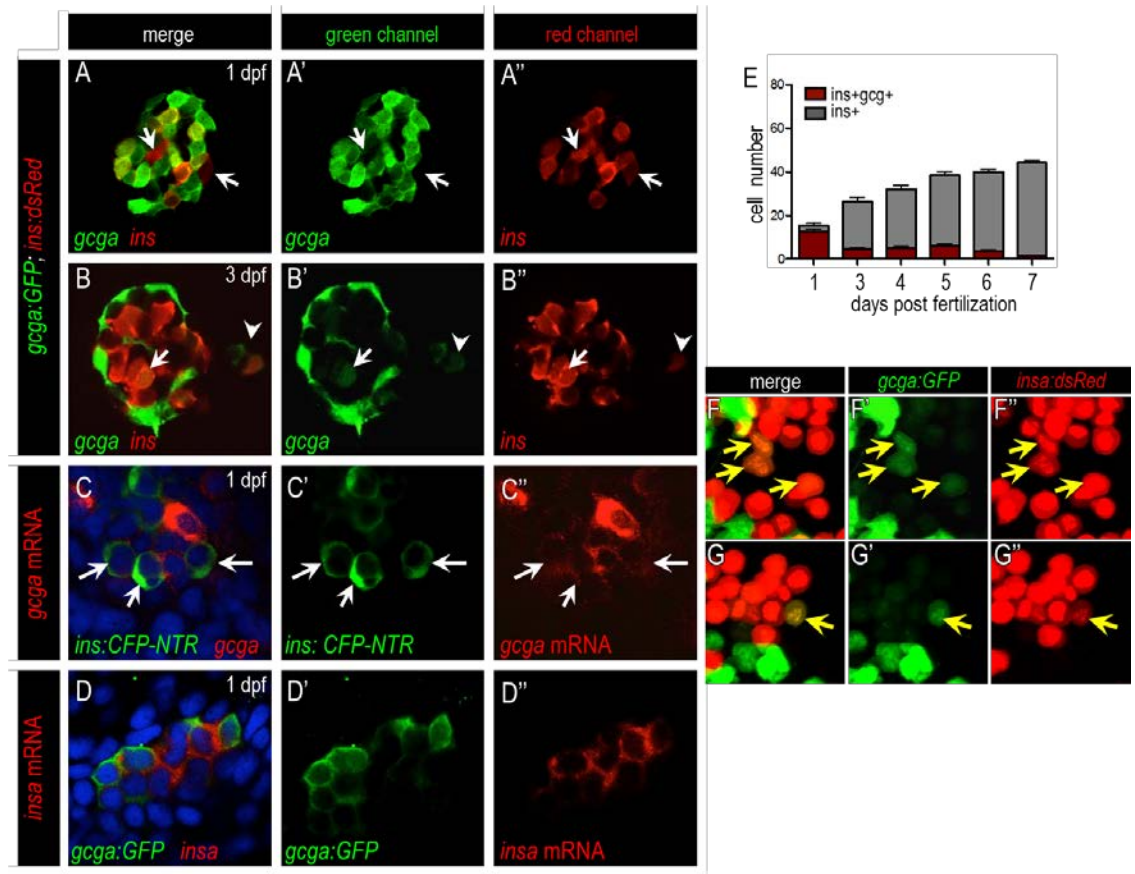


Figure 3-10. *ins*<sup>+</sup> *gcga*<sup>+</sup> cells during early islet development. (A) Confocal planes of 1 dpf *Tg(gcga:GFP); Tg(ins:dsRed)* islet showed that the majority of *ins*<sup>+</sup> cells at 1 dpf are also *gcga*<sup>+</sup>. Single positive *insulin* expressing cells are indicated by arrows. (B) Confocal planes of 3 dpf *Tg(gcga:GFP); Tg(ins:dsRed)* islet showed *ins*<sup>+</sup> *gcga*<sup>+</sup> cells in the principal islet (arrow) and *ins*<sup>+</sup> *gcga*<sup>+</sup> newly formed  $\beta$  cells (arrowhead) in the extrapancreatic duct. (C-D) Merged and single channel confocal planes of fluorescent in situ hybridization (C) *proglucagon* (red) expressed in *ins*<sup>+</sup> cells (green) at 1 dpf in *Tg(ins:CFP-NTR)* islets immunostained for GFP (green). (D) *insulin* (red) expressed in *gcga*<sup>+</sup> cell (green) in 1 dpf *Tg(gcga:GFP)* islet immunostained for GFP (green). (E) Quantification of *ins*<sup>+</sup> *gcga*<sup>+</sup> and *ins*<sup>+</sup> cells at 1 dpf through 7 dpf. *Tg(gcga:GFP); Tg(ins:dsRed)* islets showed a decrease of dual hormone-expressing cells with islet maturation.

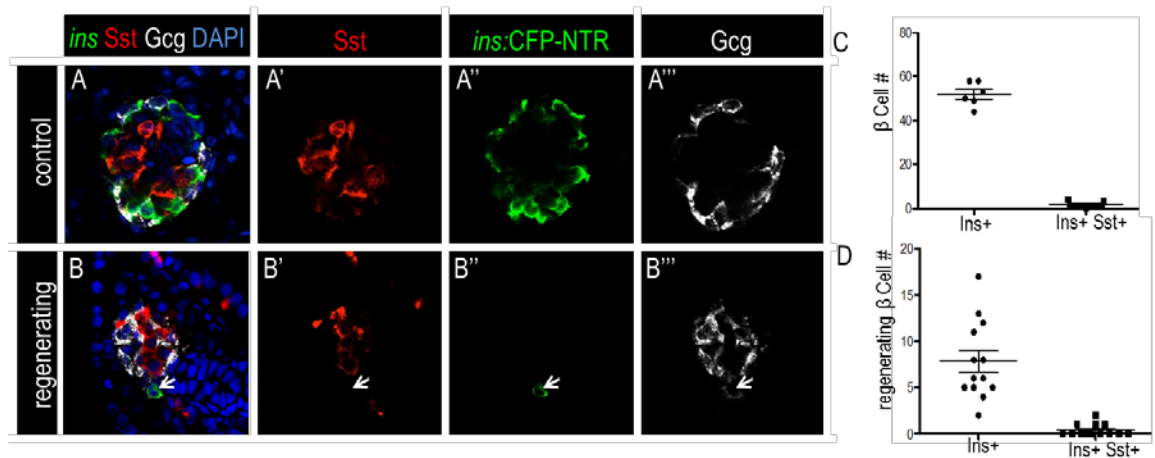


Figure 3-11. Insulin and somatostatin are rarely co-expressed in non-ablated or regenerating islets. (A-B''') Merged and single channel confocal images of 4 dpf *Tg(ins:CFP-NTR)* islets stained for CFP (green), somatostatin (red), and glucagon (white), that were not ablated (A), or ablated from 2-3 dpf (B). Regenerating *insulin*<sup>+</sup>  $\beta$  cells (arrow) were rarely labeled. (C,D) Quantification of  $\beta$  cells and regenerated  $\beta$  cells that were *insulin*<sup>+</sup> or *insulin*<sup>+</sup> somatostatin<sup>+</sup>.

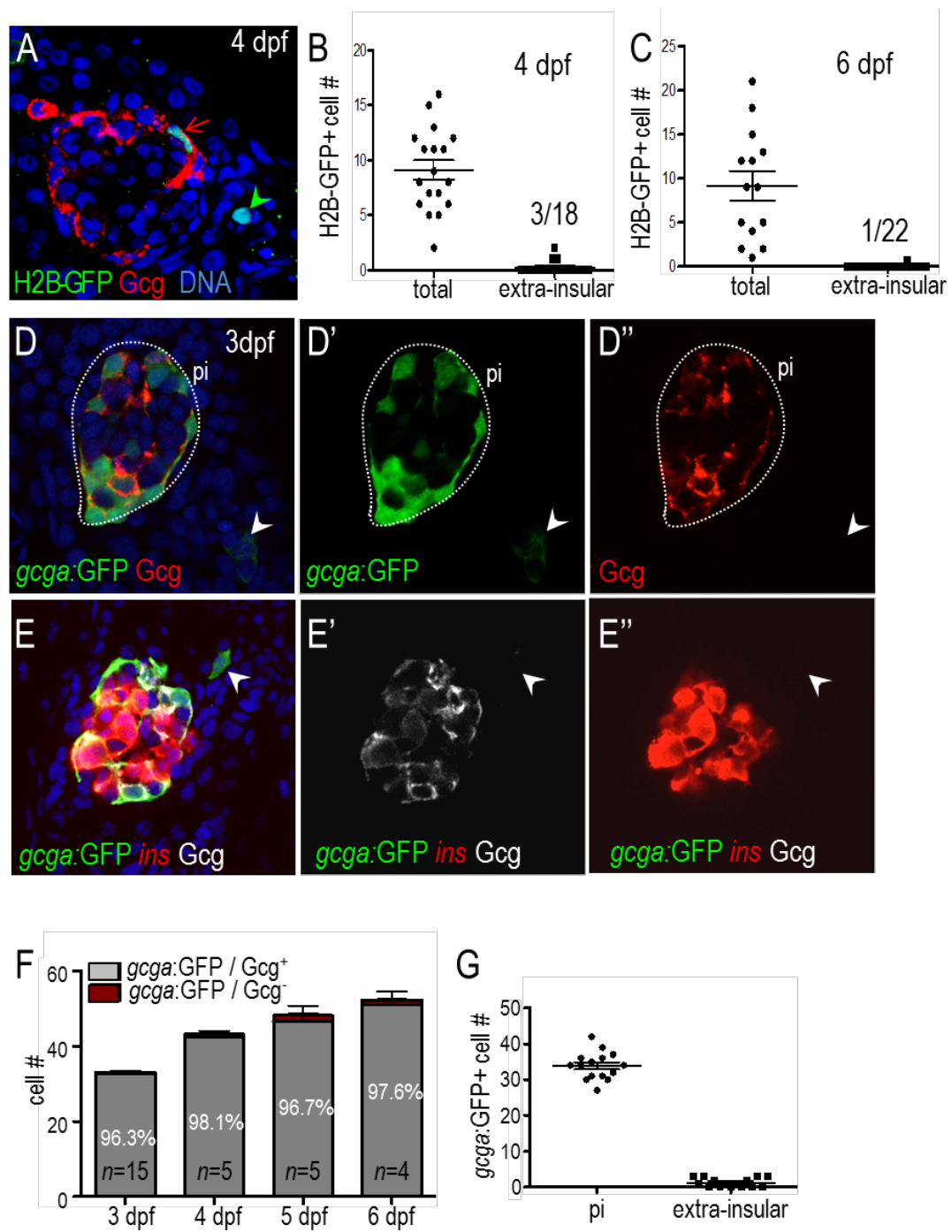


Figure 3-12. Characterization of *glucagon* promoter activity in extra-insular endocrine cells. (A) Confocal Plane of 4 dpf *Tg(gcga:cre); Tg(hs:CSH)* islet heat shock at 3 dpf stained for Glucagon (red) and GFP (green). Green arrow head indicates Glucagon-H2B-GFP<sup>+</sup> Cells outside the islet. (B,C) Quantification of total and extra-insular H2B-GFP<sup>+</sup> cells in *Tg(gcga:cre);Tg(hs:CSH)* larvae heat shocked at 3 dpf. At 4 dpf (B), 3 out of 18 samples were found to have extra-insular H2B-GFP<sup>+</sup> cells. At 6 dpf (C), 1 out of 22 samples was found to have extra-insular H2B-GFP<sup>+</sup> cells. (D) Merged and single channel confocal planes of 3 dpf *Tg(gcga:GFP)* islets stained for GFP (green) and glucagon (red). Arrowhead indicates *gcga:GFP*<sup>+</sup> glucagon<sup>-</sup> cells in pancreatic duct region outside of principal islet. (E) Merged and single channel confocal planes of 3 dpf *Tg(gcga:GFP);Tg(ins:dsRed)* islets stained for GFP (green) ,dsRed (red) and Glucagon (white). Arrowhead indicates *gcga:GFP*<sup>+</sup> *ins:dsRed*<sup>-</sup>Glucagon<sup>-</sup> cells in pancreatic duct region outside of principal islet. (F) Quantification of *gcga:GFP*<sup>+</sup> glucagon<sup>-</sup> and *gcga:GFP*<sup>+</sup> glucagon<sup>+</sup> cell number in principal islet from 3 dpf through 6 dpf. The percent of *gcga:GFP*<sup>+</sup> glucagon<sup>-</sup> cells is consistent but small. (G) Quantification of insular and extra-insular *gcga:GFP*<sup>+</sup> cell numbers.

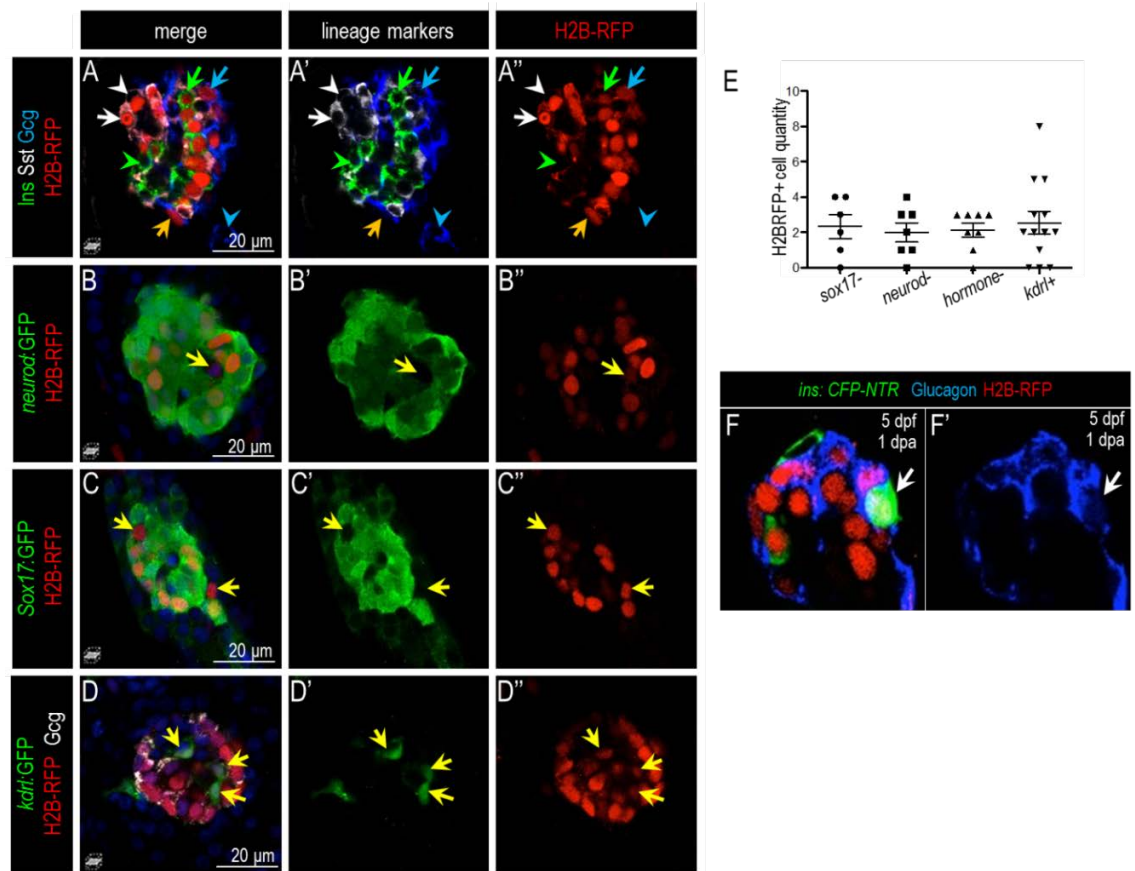




Figure 3-14. Proliferation of  $\alpha$  cells but not  $\beta$  cells during  $\beta$  cell regeneration. (A) Quantification of  $\alpha$  cell number between 5 dpf and 20 dpf in control (gray) and regenerating (red) islets ( $n \geq 3$ ). (B-C) Confocal planes of non-ablated (B) or 1 dpa (C) 5 dpf *Tg(ins:FlagNTR)* islets labeled with EdU (red) and stained for Glucagon (green). White arrows indicate Glucagon<sup>+</sup> EdU<sup>+</sup> cells in 1 dpa islet. (D-I) Confocal projections of *Tg(ins:CFP-NTR)* islets stained for phospho-histone H3 (green), glucagon (red), insulin (blue) and DNA (white) that were not ablated (D-F) or ablated from 3-4 dpf and regenerating (G-I). Islets were analyzed at 5 dpf (D,G), 6 dpf (E,H), and 7 dpf (F,I). No phospho-histone staining was observed in any non-ablated islet ( $n=21$ ) and 17.6% of regenerating islets showed staining ( $n=17$ ). (J-K) Confocal planes of non-ablated (J) or 1 dpa (K) 5 dpf *Tg(ins:CFP-NTR)* islets labeled with EdU (red) and stained for GFP (green). (L) Quantification of *ins*<sup>+</sup> EdU<sup>+</sup> cells in intact and ablated 5 dpf islets. Two-way ANOVA was used in A and Student's t-test was used in L for statistical analysis.



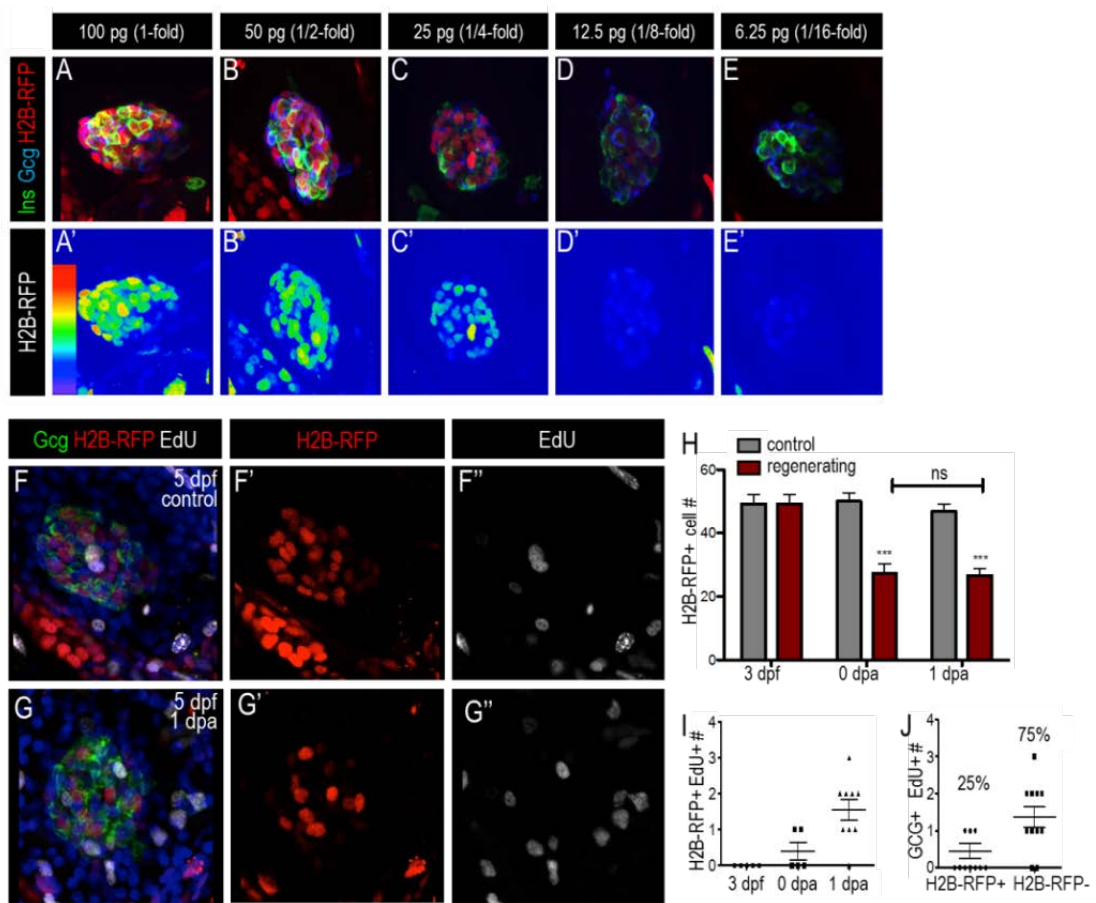
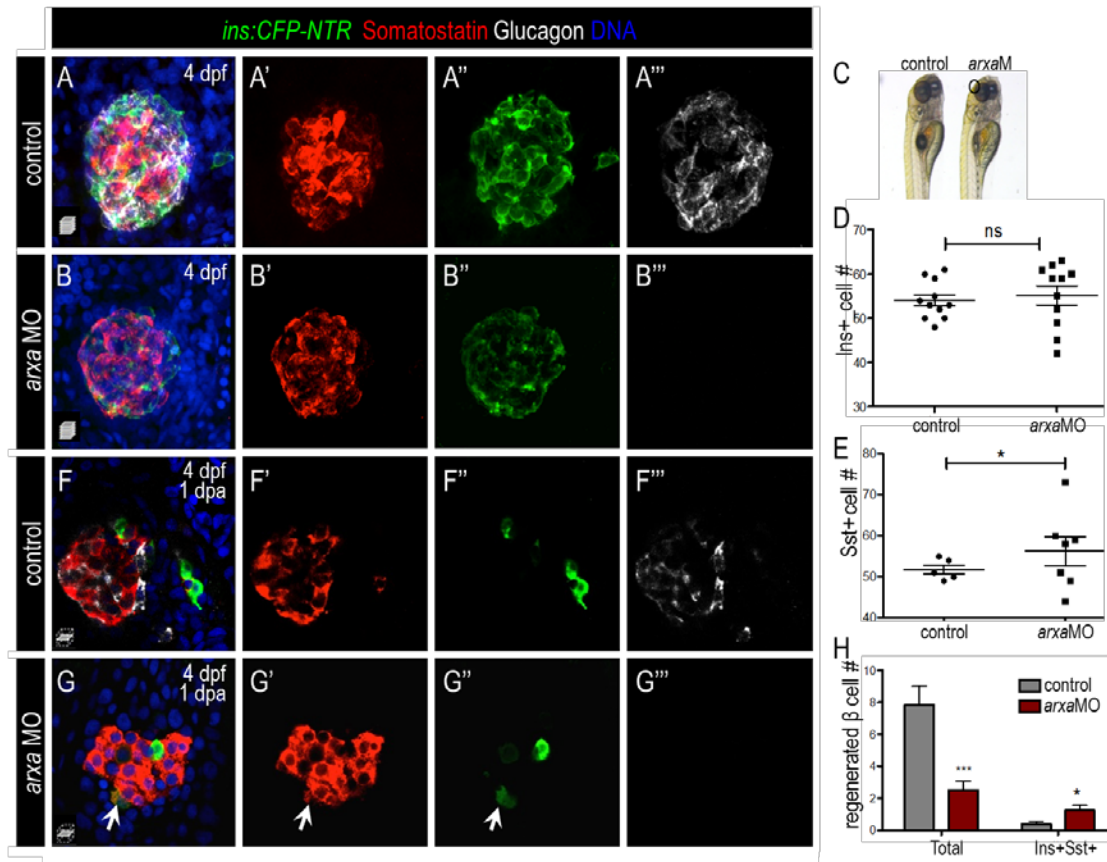




Figure 3-15. Proliferation of H2B-RFP<sup>+</sup> cells during regeneration does not dilute H2B-RFP mRNA. (A-E) Confocal projections of 5 dpf islets injected with decreasing amounts of *H2B-RFP* (red) mRNA that were immunostained for insulin (green) and glucagon (blue) to show endocrine differentiation. (A'-E') heat map representation of H2B-RFP fluorescence from confocal projections in A-E. Note that there is detectable H2B-RFP signal when mRNA was injected at the dose of 12.5 pg/embryo, which is an eightfold dilution. (F-G) Confocal projections of *H2B-RFP* mRNA injected 5 dpf control (F) and 1 dpa regenerating (G) islets that were labeled with EdU (white) for 1 hour, and stained for glucagon (green), RFP (red), and DNA (blue). (H) Quantification of total H2B-RFP<sup>+</sup> cells in islets from 3 dpf to 5 dpf in both intact islet (grey) and ablated islets (red). There is a significant decrease of total H2B-RFP<sup>+</sup> cell quantity in the islet during  $\beta$  cell ablation, but no difference of total H2B-RFP<sup>+</sup> cell number in the islet during regeneration between 0 dpa and 1 dpa. (I) Quantification of H2B-RFP<sup>+</sup> Edu<sup>+</sup> cell number during regeneration showed there are at most 2 Edu<sup>+</sup> H2B-RFP<sup>+</sup> cells per islet during regeneration. (J) Quantification of H2B-RFP<sup>+</sup> and H2B-RFP<sup>-</sup> Glucagon<sup>+</sup> Edu<sup>+</sup> cell number in 1 dpa regenerating islets showed that 75% of proliferating  $\alpha$  cells were H2B-RFP<sup>-</sup>. Two-way ANOVA was used in H for statistical analysis.



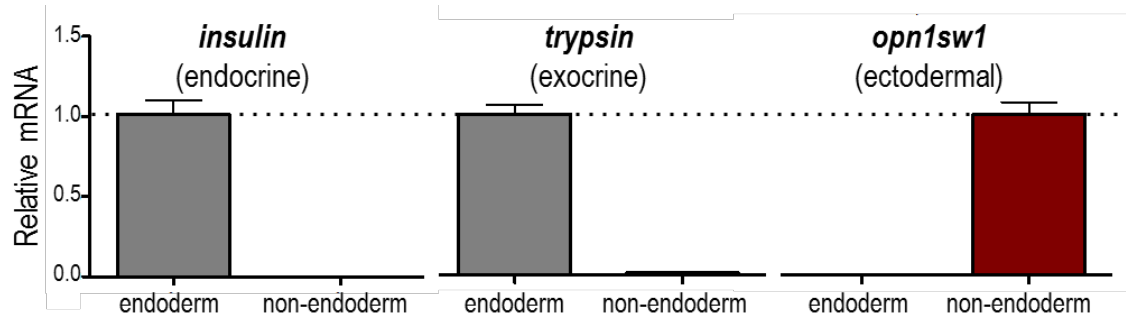


Figure 3-17. Endodermal organ cDNA preparations are enriched for endoderm. Quantitative PCR analysis on cDNA isolated from pools of dissected endodermal organs (pancreas, liver, intestine) and non-endodermal organs (the remainder of the larva). Preparations were tested for markers of the endocrine pancreas (*insa*), the exocrine pancreas (*trypsin*), and a non-endodermal opsin (*opn1sw1*). Virtually all of the *insa* and *trypsin* signal was limited to the endodermal fraction, while *opn1sw1* was restricted to the non-endodermal fraction. *insa* and *trypsin* were normalized to the expression level in endoderm, while *opn1sw1* was normalized to non-endoderm.  $n=6$  for all samples.

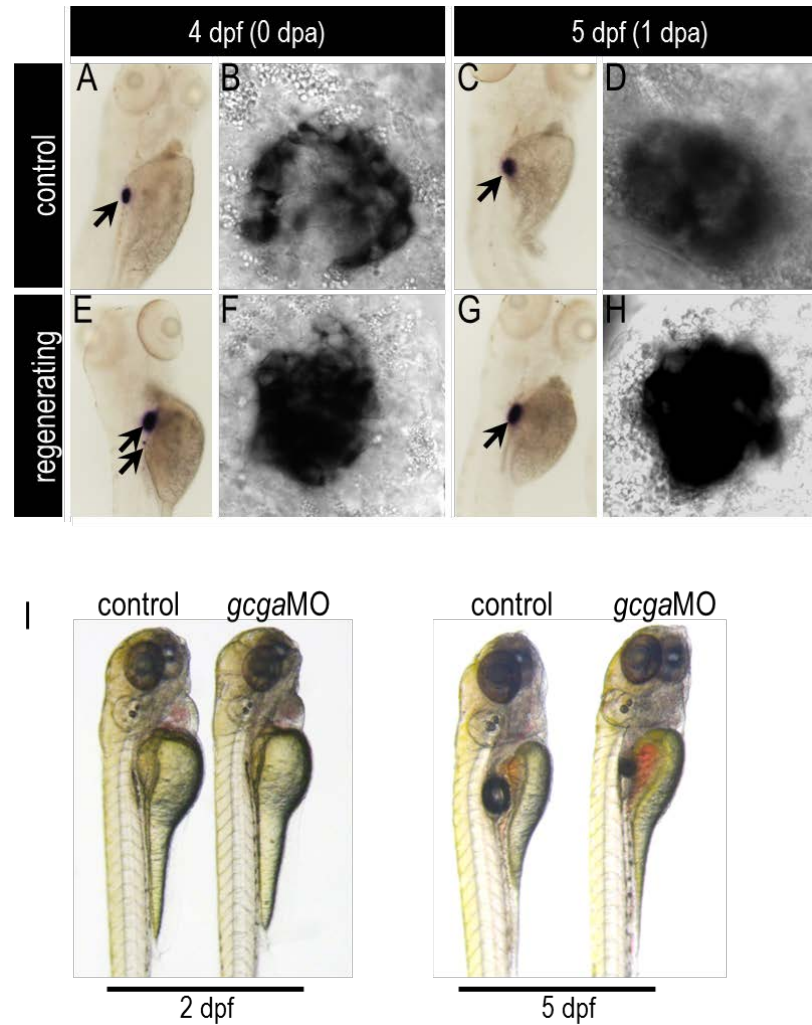


Figure 3-18. *glucagon* expression is increased in regenerating pancreata, but not is required for general embryonic development. Expression of *glucagon* revealed by *in situ* hybridization in non-ablated control (A-D), 0 dpa  $\beta$  cell-ablated (E,F), and 1 dpa regenerating (G,H) *Tg(ins:CFP-NTR)* islets at 4 dpf (A,B,E,F) and 5 dpf (C,D,G,H). (A,C,E,G) Arrows indicate *glucagon* expression in photomicrographs of whole larvae. (B,D,F,H) Confocal projections of islets. (I) 2 dpf embryos and 5 dpf larvae injected with control MO or *gcga* MO.

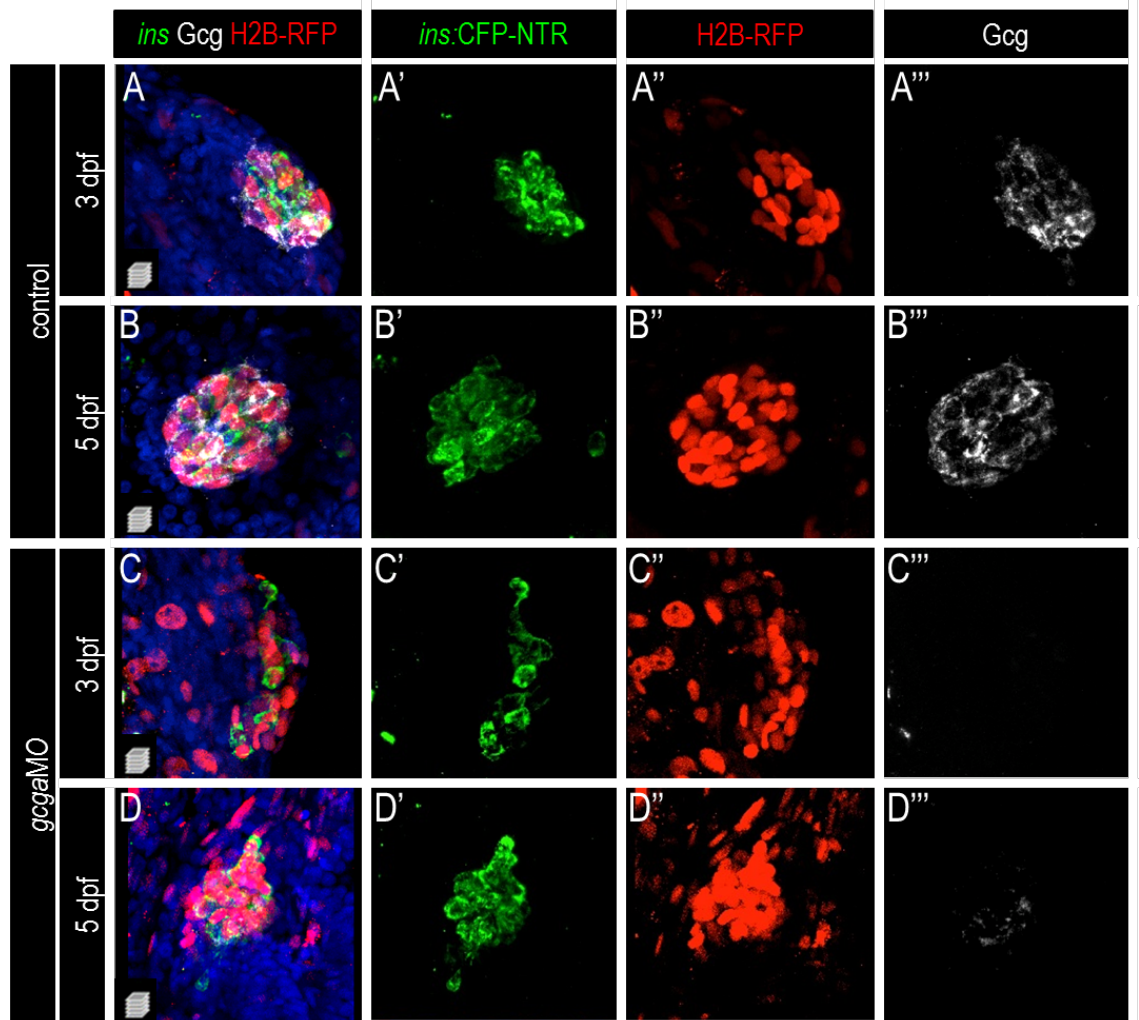


Figure 3-19. *gcga* knockdown decreases ventral pancreatic bud-derived  $\beta$  cell differentiation. (A-D''') Merged and single channel confocal projections of *Tg(ins:CFP-NTR)* islets injected with *H2B-RFP* mRNA alone (A-B''') or *H2B-RFP* mRNA + *gcga* MO that were stained for glucagon (white) ,CFP (green) and DNA (blue) at 3 dpf (A,C) and 5 dpf (B,D).

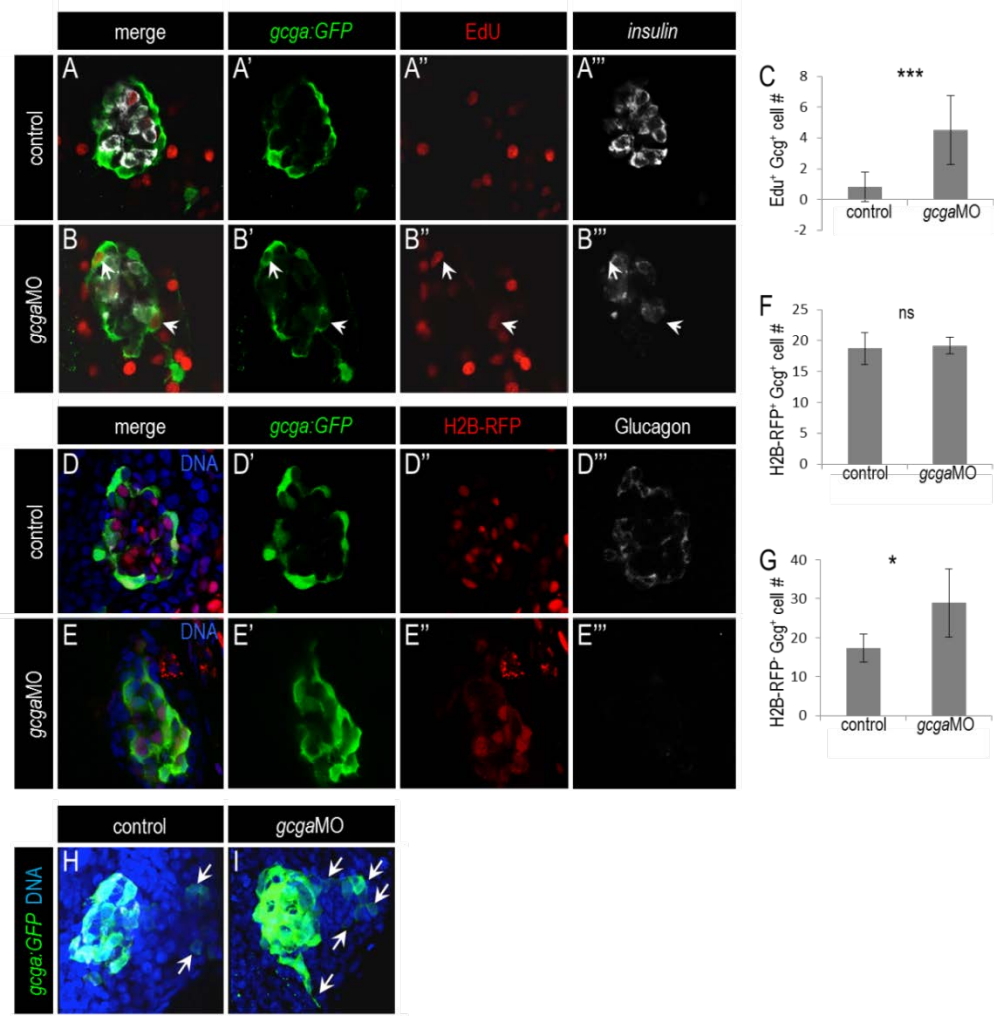


Figure 3-20. *gcga* knockdown increases  $\alpha$  cell proliferation and neogenesis. (A-B) Merged and single channel confocal planes of 4 dpf control (A) and *gcgaMO*-injected (B) *Tg(gcga:GFP)* islets labeled by insulin antibody (white) and EdU incorporation (red). (C) Quantification of EdU<sup>+</sup> *gcga:GFP*<sup>+</sup> proliferating  $\alpha$  cells in control ( $n=10$ ) and *gcgaMO* injected ( $n=12$ ) islets. (D,E) Merged and single channel confocal planes of 3 dpf control (D) and *gcgaMO*-injected (E) *Tg(gcga:GFP)* islets that were injected with H2B-RFP mRNA and labeled by Glucagon antibody (white). (F,G) Quantification of H2B-RFP<sup>+</sup> *gcga:GFP*<sup>+</sup> dorsal bud-derived  $\alpha$  cells (F) and H2B-RFP<sup>-</sup> *gcga:GFP*<sup>+</sup> ventral bud-derived  $\alpha$  cells (G) in control ( $n=7$ ) and *gcgaMO* ( $n=7$ ) islets. (H-I) Confocal projections of 3 dpf control (H;  $n=7$ ) and *gcgaMO*-injected (I;  $n=7$ ) *Tg(gcga:GFP)* islets. Arrows indicate newly forming *gcga:GFP*<sup>+</sup> cells derived from pancreatic ducts.

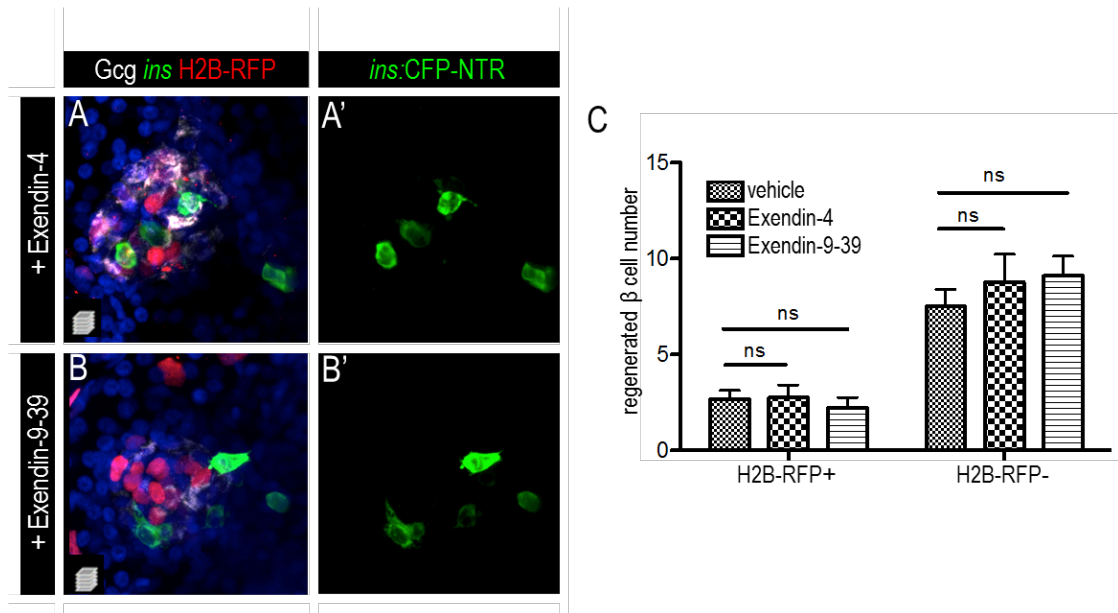


Figure 3-21. Glp-1 receptor agonist or antagonist treatment does not affect  $\beta$  cell regeneration. (A-B') Merged and single channel confocal projections of 5 dpf regenerating 1 dpa *H2B-RFP* mRNA injected islets that were treated with Exendin-4 (A) or Exendin-9-39 (B) during regeneration (comparable untreated control islets are shown in Figure 4). (C) Quantification of H2B-RFP<sup>+</sup> and H2B-RFP<sup>-</sup> regenerated  $\beta$  cells in vehicle, Exendin-4, and Exendin-9-39 treated islets. Two-way ANOVA was used in C for statistical analysis.



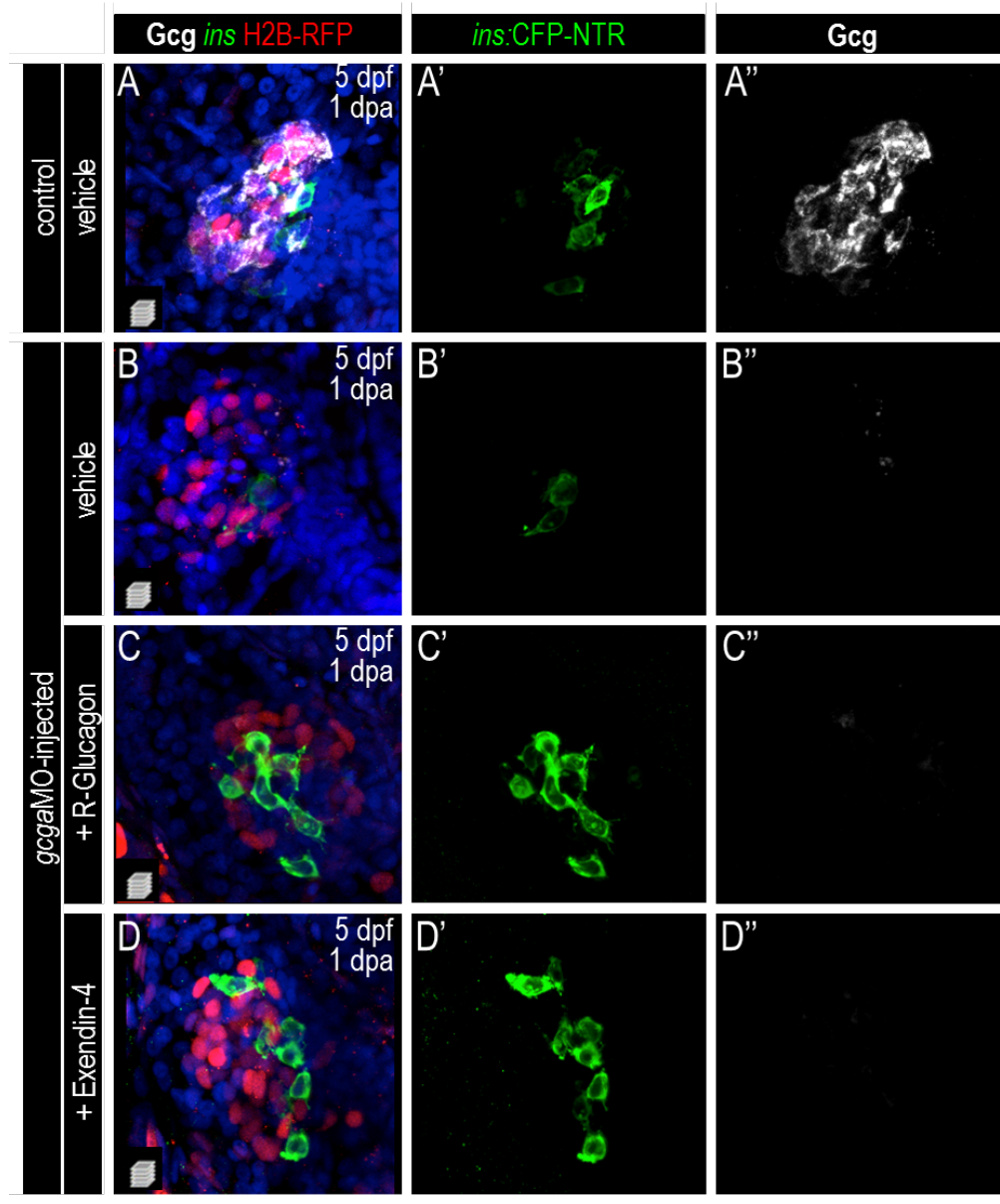


Figure 3-22. Glucagon or Exendin-4 injections rescue  $\beta$  cell regeneration in *gcga* MO-injected islets. (A-D'') Merged and single channel confocal projections of 5 dpf/1 dpa regenerating *H2B-RFP* mRNA-injected (red) islets that were not morpholino-injected (A), or injected with *gcga*MO (B-D) and stained for CFP (*ins*:CFP-NTR; green), glucagon (white), and DNA (blue).



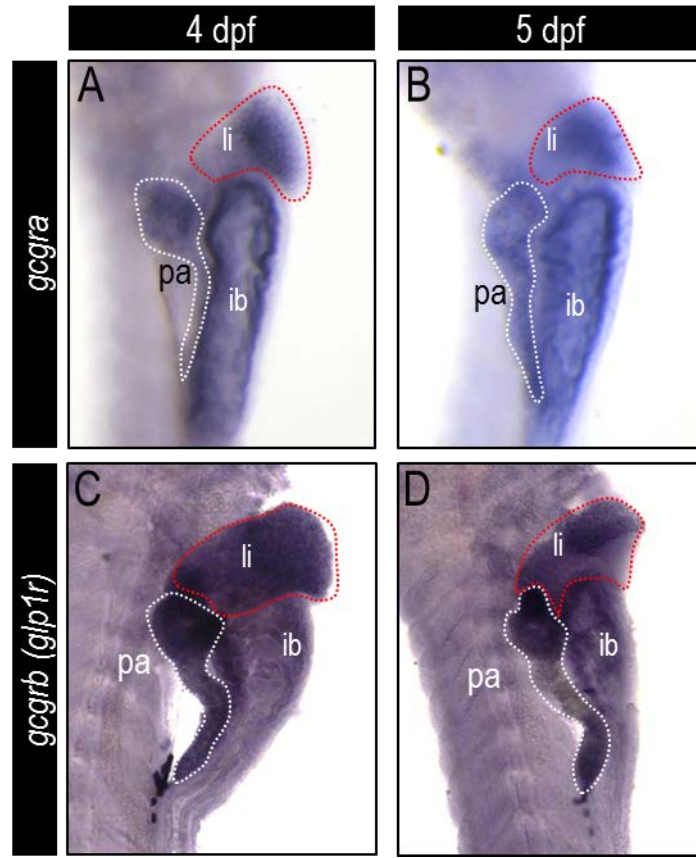


Figure 3-23. *glucagon receptor* and *glp-1 receptor* mRNAs are expressed in the larval pancreas. (A-D) Whole mount *in situ hybridization* showing regionally restricted expression of glucagon receptor (*gcgra*; A,B) and glp-1 receptor (*gcgrb/glp1r*; C,D) in the endodermal organs of 4 dpf (A,C) and 5 dpf (B,D) larvae. Note that both receptors are expressed throughout the pancreas, including exocrine and islet regions. Abbreviations: pa: pancreas; li: liver; ib: intestinal bulb.

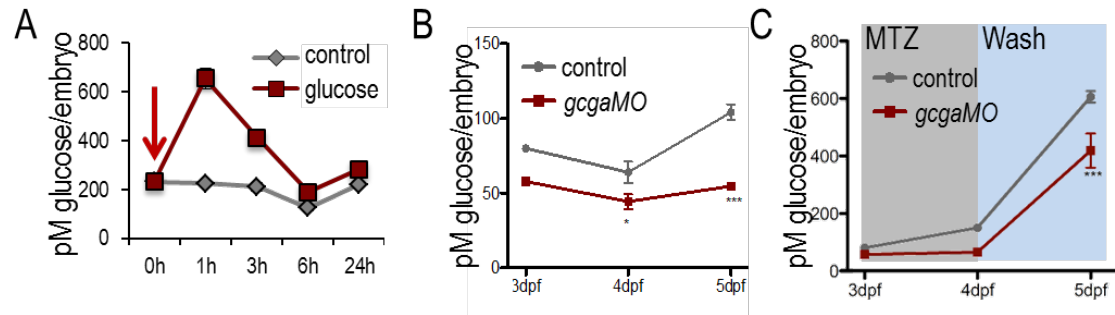


Figure 3-24. *glucagon* gene products regulate free glucose levels in zebrafish. (A) Free glucose levels measured periodically after mannose control (gray line) or glucose (red line) injection (arrow). (B) Free glucose measurement of control (gray line) and *gcga MO*-injected (red line) larvae at 3, 4 and 5 dpf. (C) Free glucose measurement in control uninjected and *gcgaMO*-injected *Tg(ins:CFP-NTR)* larvae in which  $\beta$  cells were ablated from 3 to 4 dpf. In both B and C, morpholino-injected zebrafish have diminished glucose levels. Two-way ANOVA was used in B and C for statistical analysis.

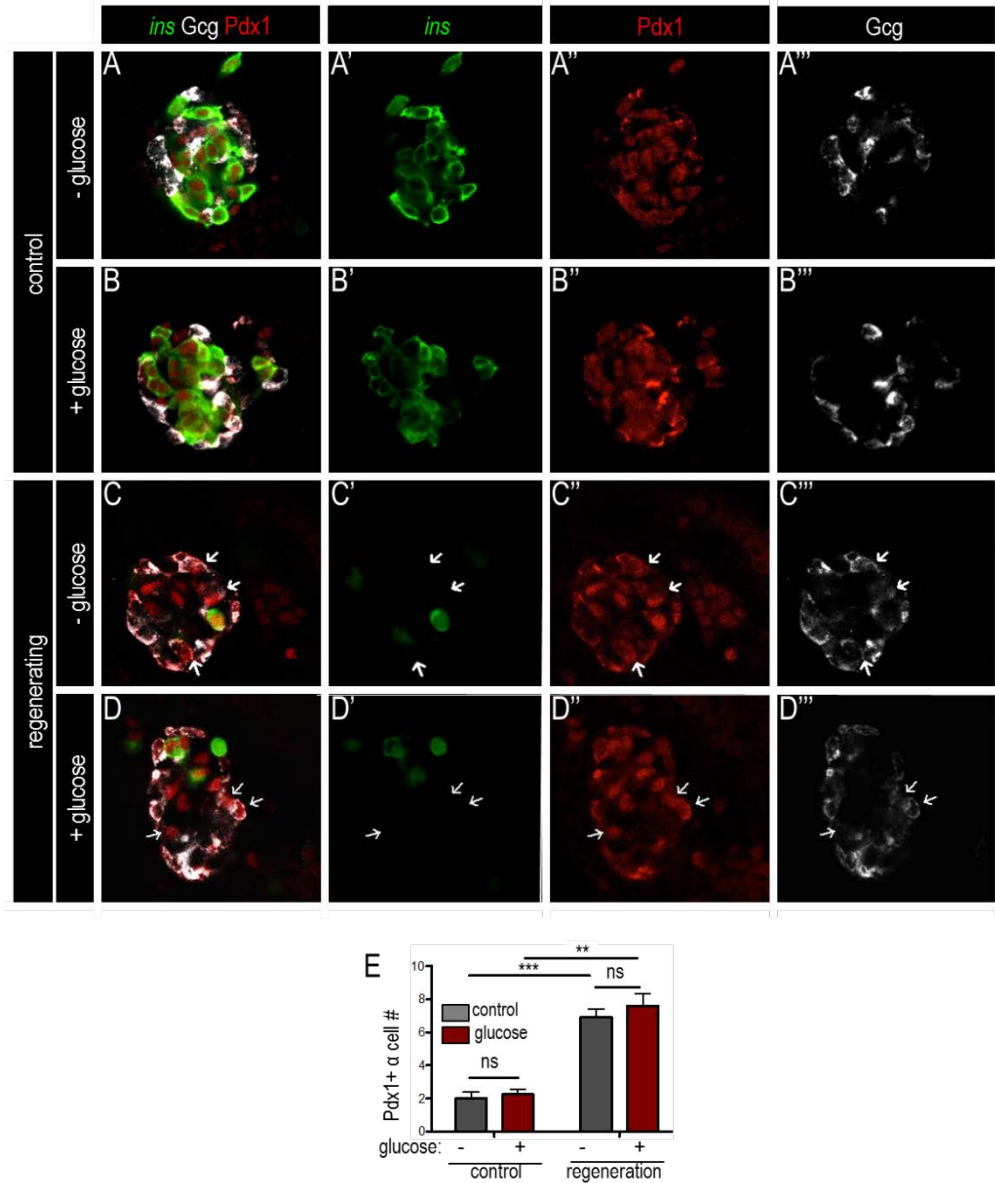


Figure 3-25. Quantity of Pdx1<sup>+</sup>  $\alpha$  cells is not affected by glucose injection. (A-D''') Merged and single channel confocal projections of 5 dpf non-ablated control (A-B''') and 1 dpa regenerating (C-D''') *Tg(ins:CFP-NTR)* islets that were not injected (A,C) or were injected with glucose (B,D) at 4 dpf. Islets were stained for Pdx1 (red), CFP (*ins:CFP-NTR*; green), and glucagon (white). Pdx1<sup>+</sup> glucagon<sup>+</sup> double positive cells are marked with white arrows in C,D. (E) Quantification of Pdx1 expression in  $\alpha$  cells of intact and regenerating larvae was not altered by glucose injection; control group ( $n=8$ ) and glucose treated group ( $n=12$ ). Two-way ANOVA was used in E for statistical analysis.

Table 3-1. Pancreatic gene expression in regenerating endoderm.

age		gene						
		<i>insa</i>	<i>gcga</i>	<i>arx</i>	<i>mafa</i>	<i>mafb</i>	<i>neurod</i>	<i>ela3l</i>
<b>0</b> <b>dpa</b>	<b>4</b> <b>dpf</b>	-22.08**	+1.577**	-1.262	-1.335*	-1.801**	-1.189	-1.010
<b>1</b> <b>dpa</b>	<b>5</b> <b>dpf</b>	-39.43***	+2.00***	-1.317	+1.682***	-1.275	-1.324	+1.054
<b>3</b> <b>dpa</b>	<b>7</b> <b>dpf</b>	-10.45***	-1.15	-1.243	+1.686***	+1.022	+1.262*	-1.241*
Positive and negative value indicates fold increase or decrease of gene expression in regenerating endoderm compared with age matched un-ablated control. MTZ was added from 3 dpf to 4 dpf for ablation of $\beta$ cells. $n=3$ for each group. Two way ANOVA analysis * $p \leq 0.05$ , ** $p \leq 0.01$ , *** $p \leq 0.001$								

## **CHAPTER FOUR: An insulin signaling feedback loop regulates pancreas progenitor cell differentiation during islet development and regeneration.**

(As submitted for publication with Robertson, M. A.; and Mastracci, T.M., 2015)

### **IV.A. Summary**

As one of the key nutrient sensors, insulin signaling plays an important role in integrating environmental energy cues with organism growth. In adult organisms, relative insufficiency of insulin signaling induces compensatory expansion of insulin-secreting pancreatic  $\beta$  ( $\beta$ ) cells. However, little is known about how insulin signaling feedback might influence neogenesis during the establishment of  $\beta$  cell mass during embryonic development. Here, using genetic approaches and a unique cell transplantation system in developing zebrafish, we have uncovered a novel role for insulin signaling in negatively regulating the differentiation of pancreatic progenitors. Blocking insulin signaling in pancreatic progenitors hastened the expression the essential  $\beta$  cell genes *insulin* and *pdx1*, and promotes  $\beta$  cell fate at the expense of  $\alpha$  cell fate. This indicates that insulin signaling constitutes a tunable mechanism for  $\beta$  cell compensatory plasticity during early development. Moreover, using a novel blastomere-to-larva transplantation strategy, we found that loss of insulin signaling in the endoderm-committed blastomeres drove their differentiation into  $\beta$  cells. Furthermore, the extent of this differentiation was dependent on the function of the  $\beta$  cell mass of the host. Altogether, our results indicate that modulation of insulin signaling will be crucial for the development of  $\beta$  cell restoration therapies for diabetics; further clarification of the mechanisms of insulin signaling in  $\beta$  cell progenitors will reveal therapeutic targets for both in vivo and in vitro  $\beta$  cell generation.

### **IV.B. Introduction**

Insulin is a crucial gluco-regulatory peptide hormone produced by pancreatic  $\beta$  cells that is released in proportion to levels of circulating glucose. Under conditions of fluctuating metabolic demands and energy availability, the effective functional  $\beta$  ( $\beta$ ) cell

mass (insulin releasing capacity of the pancreas) is regulated to match physiological demands through  $\beta$  cell compensation. While compensation can be transiently mediated in part via increased insulin production and release from existing  $\beta$  cells, long term  $\beta$  cell compensation involves the expansion of  $\beta$  cell mass by multiple mechanisms (36,209,304). For instance, physiological stresses like over-nutrition and pregnancy can accelerate  $\beta$  cell replication (305-307). Additionally,  $\beta$  cells may arise via neogenesis from non-  $\beta$  cell sources, including differentiation from facultative progenitor cells and conversion from other pancreatic endocrine cells (160,161,170,171,176,308), though much remains to be discovered about the mechanisms regulating this process. The failure of  $\beta$  cell compensation to meet insulin demand will result in diabetes mellitus, a metabolic disease of insufficient insulin signaling that is characterized by uncontrolled hyperglycemia and its associated morbid complications. A comprehensive understanding of molecular mechanisms that sense insulin insufficiency and translate it into  $\beta$  cell compensation responses will impact the design of better diabetes therapies.

Several growth factors and cytokines have been shown to regulate  $\beta$  cell replication in response to metabolic demand. In the adult islet, insulin secreted by  $\beta$  cells appears to feedback upon  $\beta$  cells to regulate islet size and  $\beta$  cell mass (309). Activation of insulin receptor triggers its autophosphorylation, which is followed by downstream signal propagation via the key effector *Insulin Receptor Substrates 1/2* (IRS1/2) to the Akt and Mitogen Activated Protein Kinase (MAPK) pathways; these mediate many growth and metabolism responses (310). Knockout of insulin receptors in  $\beta$  cells ( $\beta$ IRKO) abolished compensatory  $\beta$  cell mass expansion in adult mice and resulted in hyperglycemia (311). Further, this suggests that reported influences of glucose on  $\beta$  cell replication may be due in part to the indirect effects of augmenting insulin secretion (312). Although much is known about replication-induced  $\beta$  cell mass compensation, little is known regarding the cellular and molecular mechanisms of neogenesis induced  $\beta$  cell mass compensation.

It is likely that some mechanisms regulating  $\beta$  cell compensation via neogenesis are common to both the mature and developing pancreas, and the embryo is an especially amenable system in which to study  $\beta$  cell formation. However, while the intrinsic developmental programs regulating endocrine differentiation have been very well characterized (313), the extrinsic signals that control induction and differentiation of  $\beta$

cells, and match  $\beta$  cell mass to the needs of the embryo are less well understood. Among the pathways studied are FGF and Notch signaling, which suppress differentiation of pancreas progenitors (99,106,107) and EGF signaling, which influences  $\beta$  cell neogenesis (314-316). Interestingly, little is known about the roles of the pancreatic hormones during development. While glucagon signaling regulates  $\alpha$  ( $\alpha$ ) cell mass by proliferation, neogenesis, and fate switching mechanisms (139,141,284), it is not clear whether other islet hormones like insulin have a significant role in the acquisition and stability of cell fates in the developing islet. Even though the insulin signaling pathway has been deeply studied using mouse knockout models, the results from previous developmental studies appear contradictory. Mice lacking insulin receptor exhibit severe hyperglycemia at birth despite grossly normal islets (317-319). However, knockout of either or both of the mouse *insulin* orthologues (320) or downstream effectors like Akt lead to marked islet hyperplasia (321). Therefore, further investigations of the roles of insulin in other model systems may help resolve how insulin signaling regulates  $\beta$  cell neogenesis during development as well as in pathologies like diabetes.

Zebrafish are a relevant and powerful system in which to study  $\beta$  cell formation and homeostasis: they share key features of their carbohydrate metabolism and their  $\beta$  cell differentiation program with mammalian systems (221), while they also afford many experimental advantages (322). As in mice and humans, the zebrafish pancreas arises from two Pdx1-expressing progenitor domains that fuse to establish the architecture of the pancreas (226,267,323). In zebrafish, the dorsal bud appears at approximately 14 hours post fertilization (hpf) and gives rise to endocrine cell types, which then cluster into the principal islet by 24 hpf. In contrast, the ventral bud emerges from the endoderm around 34 hpf, then migrates towards, and engulfs the principal islet while differentiating into both exocrine and endocrine lineages. In this study, we have used zebrafish as a model system to explore the role of insulin signaling on  $\beta$  cell generation during development. Using genetic approaches in zebrafish that either inhibit insulin production or impair activity of its downstream effector, IRS2, we show here that insulin signaling has an inhibitory role during early pancreas development: loss of insulin signaling drove the early differentiation of pancreatic progenitors into  $\beta$  cells. Moreover, using a novel blastomere-to-larva transplantation strategy, we found that loss of insulin signaling in

endoderm-committed blastomeres fostered their differentiation into  $\beta$  cells, and that the extent of this differentiation was dependent on the function of the host  $\beta$  cell mass. Our data suggest that manipulation of the insulin signaling pathway will be crucial for regenerative medicine approaches to diabetes therapies, including  $\beta$  cell differentiation from in situ progenitors during regeneration and from stem cells *in vitro*.

## IV.C. RESULTS

### Knockdown of *insulin* drives $\beta$ cell early differentiation

To determine whether pancreatic progenitor cells are competent to receive insulin signals, we first performed whole mount *in situ* hybridization to illuminate the expression of insulin receptors. There are two isoforms of zebrafish insulin receptor, insulin receptor a (*insra*) and -b (*insrb*) (250). We found that both were strongly expressed in zygotes, indicating that maternal contributions may affect early embryonic development (Figure 4-1A, Figure 4-8A-B). Only *insrb* was expressed in the embryonic pancreatic endoderm during early pancreas development, as visualized by co-localization with endoderm marker *sox17* at 48 hours post fertilization (hpf) (Figure 4-1A-C). However, both *insra* and *insrb* were expressed in the pancreas, liver and intestine in larvae at 108 hpf, which may reflect metabolic roles for insulin signaling during later developmental stages (Figure 4-1A, Figure 4-8).

In order to analyze the roles of insulin signaling during pancreatic progenitor differentiation we designed specific splice-blocking morpholinos (MOs) to knockdown *insulin* during embryogenesis (Figure 4-1D). A morpholino targeting the exon 2-intron 2 boundary was used to disrupt zygotic *insulin a* (hereafter *insa*) pre-mRNA splicing (Figure 4-9A). Injection of *insa* morpholino (*insaMO*) resulted in two aberrant splicing products of *insa* pre-mRNA, indicating efficacy of the morpholino (Figs. 4-9B-C, 4-10). Furthermore, using immunofluorescent staining, we observed a loss of Insulin protein in *insaMO*  $\beta$  cells (Figure 4-1E-F), but no general developmental defects were observed in *insaMO*-injected embryos (Figure 4-9D). Due to genomic duplication, zebrafish have a second orthologue of *insulin*, *insb* (249,320). In contrast to the mouse *Insulin* paralogues *Ins1* and *Ins2* that are expressed identically (320,324,325), the expression patterns of



zebrafish *insulin* paralogues differ significantly. While *insa* is expressed only in pancreatic  $\beta$  cells, *insb* is widely expressed, especially in the head and somatic musculature, suggesting that these two genes execute different developmental functions in zebrafish (249). To test this hypothesis, we knocked down *insb* using a morpholino that targeted the exon 4-intron 4 boundary (Figure 4-11). In *insbMO*-injected embryos we observed efficient disruption of *insb* splicing and severe developmental defects, including microcephaly and shortening of the antero-posterior axis (Figure 4-11B). However, the expression of insulin protein in the islet and the number of pancreatic  $\beta$  cells was not affected (Figure 4-11E-G).

Next, we analyzed  $\beta$  cell formation during development in *insa*-deficient embryos. For this, we considered differentiation from both sources of  $\beta$  cells: the dorsal (early) and ventral (late) pancreatic buds. We have previously shown that  $\beta$  cells derived from each of these origins can be distinguished by a label retaining cell assay (LRC) (222), in which embryos are initially uniformly labeled by zygotic injection of H2B-RFP mRNA, and the fluorescent intensity of the encoded fluorescent protein is diluted only by mitosis. The dorsal derived  $\beta$  cells (DBC), which differentiate early and become quiescent, remain H2B-RFP positive. In contrast, ventral bud-derived progenitor cells lose H2B-RFP signal via extensive proliferation before ventral  $\beta$  cell (VBC) differentiation (222). Using this approach we quantified DBCs and VBCs in *insaMO*-injected pancreata throughout development. Consistent with our previous findings (222,271), we found that only DBCs were present in the principal islet of control embryos at 24 hpf, whereas H2B-RFP<sup>+</sup> VBCs cells were detected after 48 hpf and continued to increase throughout development (Figure 4-2A-C,G,H). We found that *insaMO* did not influence DBC number, but  $\beta$  cell mass derived from the ventral bud was sharply increased (Figure 4-2D-F, G, H). Surprisingly, a significant number of H2B-RFP<sup>+</sup> VBCs were detected in *insaMO* embryos at 24 hpf (Figure 4-2A, D). Despite this early increase, we found no difference in total  $\beta$  cell number at 96 hpf between *insaMO*-injected and control groups (Figure 4-2C, F, H). These data indicate that loss of insulin expression in the early pancreatic islet drives ventral bud-derived pancreas progenitor cells to precociously differentiate into  $\beta$  cells. We examined the proliferation of  $\beta$  cells at 24 hpf using both phosphohistone H3 (PHH3) immunofluorescence and EdU DNA incorporation assays and thereby excluded the

possibility that the precocious H2B-RFP<sup>+</sup>  $\beta$  cells detected in the 24 hpf *insaMO*-injected embryos were derived from hyper-proliferative dorsal bud-derived cells. In both analyses, proliferating  $\beta$  cells were not observed in control or *insaMO*-injected pancreata at 24 hpf (Figure 4-12A-D). These data support the interpretation that the supernumerary H2B-RFP<sup>+</sup>  $\beta$  cells in *insaMO*-injected embryos arose by neogenesis from ventral bud sources rather than by proliferation of existing  $\beta$  cells or other dorsal bud-derived sources. Concordantly, in 24 hpf *insaMO-injected* pancreata, but not in control pancreata, ectopic insulin<sup>+</sup> cells were detected in the ventral endoderm (Figure 4-12E-I).

Previous studies have shown that homeodomain transcription factor Pdx1 is critical for both pancreas progenitor and  $\beta$  cell development. The pancreas is derived from a pool of multipotent progenitor cells that express Pdx1 (64,68). During differentiation, Pdx1 expression subsides in most pancreatic cellular lineages, but is enhanced in  $\beta$  cells where it is essential for  $\beta$  cell differentiation and fate maintenance (63,281,326). These roles of Pdx1 are conserved in zebrafish to regulate  $\beta$  cell formation as well as ventral pancreatic progenitor differentiation (227,282). To analyze the mechanisms underlying insulin directed  $\beta$  cell formation, we analyzed the expression of Pdx1 in both control and *insaMO*-injected embryos. Our data showed that Pdx1 expression was restricted to the principal islet in 24 hpf embryos, and after the induction of the ventral pancreatic bud at 48 hpf, Pdx1 was expressed in both the principal islet and adjacent endoderm (Figure 4-2I, J). After *insaMO* knockdown, we observed expansion of the Pdx1 domain in the principal islet region (Figure 4-2K, L, arrow), which is consistent with our interpretation that insulin loss drives excess  $\beta$  cell differentiation from ventral pancreas. Quantitative PCR further confirmed an increase in Pdx1 expression at early stages of pancreas development (24 and 48 hpf), but not during later stages (72 and 96 hpf; Figure 4-2O).

The loss of insulin signaling due to *insaMO* not only expanded *pdx1* expression in the principal islet, but also increased *pdx1* expression in the adjacent endoderm (Figure 4-2K-L, arrowheads), from which the ventral pancreatic progenitor cells are derived. We next investigated how loss of insulin affected induction of the ventral pancreatic bud by analyzing the co-expression of Pdx1 and Ptf1a, two transcription factors that together define the ventral pancreatic progenitor domain (68). This domain was marked by immunofluorescent staining of Pdx1 in *Tg(ptf1a:EGFP)<sup>jh1</sup>* embryos. At 41 hpf, we found

that all *Ptf1a*<sup>+</sup> cells in the ventral endoderm were also *Pdx1*<sup>+</sup>, suggesting that they are the equivalent of mammalian pancreatic progenitor cells (Figure 4-2M), and is in accord with *ptf1a* lineage tracing studies in zebrafish (304). Surprisingly, in contrast to the expansion of *Pdx1* domain observed with *insaMO* knockdown, we observed that pancreatic *ptf1a* expression was decreased after *insaMO* knockdown, as was the number of *Pdx1*<sup>+</sup> *ptf1a*<sup>+</sup> ventral pancreatic progenitors (Figure 4-2N, P). These findings indicate that knockdown of insulin induces early differentiation of  $\beta$  cells from ventral pancreatic progenitors, and consequently diminishes the ventral pancreatic progenitor pool. In accord with this finding, we also observed decreased of total pancreatic size in *insaMO* injected larvae (date not shown). Overall, our findings suggest that insulin signaling is part of a feedback loop by which newly functional  $\beta$  cells modulate the continued differentiation of pancreatic progenitors. As such, the loss of insulin can induce a compensatory increase of  $\beta$  cell differentiation during early islet development (Figure 4-12J).

### **Intracellular blockade of insulin signaling induces precocious $\beta$ cell differentiation.**

In order to further investigate the influence of insulin signaling on  $\beta$  cell differentiation, we generated a truncated mutant form of zebrafish IRS2 that retains the N-terminal pleckstrin homology and phosphotyrosine binding domains, but substitutes GFP for the C-terminal SH2-binding domains (Figure 4-S6A). As such, this construct (dnIRS2-GFP), is expected to act as a dominant negative regulator of insulin signaling as seen with similar constructs [4,69,70]. Indeed, when *dnIRS2-GFP* mRNA was transcribed *in vitro* and injected into zygotes, we observed that dnIRS2-GFP protein was enriched at the plasma membrane in 5 hpf embryos (Figure 4-3A-B) and remained expressed in the embryos through 24 hpf (Figure 4-13B). To determine whether this mode of insulin signaling blockade affected the formation of  $\beta$  cells, we examined the expression of *pdx1* and insulin in *dnIRS2-GFP* mRNA-injected embryos at 24 hpf. Relative to controls, we observed a marked increase in *pdx1* expression in the pancreatic region and a 32% increase in the number of *ins*<sup>+</sup>  $\beta$  cells marked by *Tg(insa:dsRed)* (Figure 4-3E-G). Concordantly, treatment with the PI3 kinase inhibitor wortmannin, which should block some aspects of downstream insulin signaling (308), also resulted in

increased  $\beta$  cell formation in *Tg(insa:CFP-NTR)* embryos (Figure 4-3H, Figure 4-14A-F). In contrast, blockade of the MAPK pathway via the Erk inhibitor U0126 did not significantly influence  $\beta$  cell number (Figure 4-14G). Taken together, these results suggest that early  $\beta$  cell formation from progenitors may be primarily regulated via the PI3K branch of the insulin signaling pathway.

Next, we generated a conditional transgenic model to knockdown of insulin signaling through temporally defined misexpression of *dnIRS2-GFP*. This Cre recombinase-switchable, heat shock-inducible transgenic line, *Tg(hs:loxp-mcherry-loxp-stop-dnIRS2-GFP;cryaa:CFP)*, was crossed with *Tg(hs:Cre)* (327) to generate double transgenic embryos in which *dnIRS2-GFP* is globally inducible by heat shock, hereafter *dnIRS2* (Figure 4-13C). We verified the efficiency of insulin signaling blockade in *dnIRS2* embryos using immuno-blot for phospho-Akt protein that was extracted from 54 hpf control and *dnIRS2* embryos after heat induction. These *dnIRS2* transgenic embryos showed a 65% decrease in phospho-Akt as compared with controls (Figure 4-13D). Using this model, we then analyzed how Pdx1 expression responded to *dnIRS2* over-expression. In control 24 hpf embryos, Pdx1 was expressed predominantly in dorsal endocrine cells and weakly in adjacent endoderm (Figure 4-3I). However, in *dnIRS2* embryos, Pdx1 expression was strongly increased in both the principal islet (arrows) and the adjacent pre-pancreatic endoderm (Figure 4-3J, arrowheads). Furthermore, at 48 hpf, *dnIRS2* embryos showed an increased *pdx1* expression pattern that was similar to that of *insaMO*-injected embryos (Figure 4-13E). Next, we analyzed  $\beta$  cell development in *dnIRS2* embryos. As with *insulin* knockdown, we found increased  $\beta$  cell formation in *dnIRS2* transgenic embryos (Figure 4-3K-M). Interestingly, we also noted a significant decrease in the number of glucagon<sup>+</sup>  $\alpha$  cells (Figure 4-3K-M). Together, our results indicate that insulin signaling blockade induces compensatory  $\beta$  cell differentiation from progenitors within the ventral pancreatic bud. In addition, our data suggest that insulin signaling also influences the specification of non- $\beta$  endocrine subtypes in the islet.

**Knockdown of *insulin* induces ectopic Pdx1 expression in  $\alpha$  cells and destabilizes  $\alpha$  cell fate**

To examine how insulin signaling affects  $\alpha$  cell fate specification during development, glucagon expression was examined in control and *insaMO*-injected *Tg(insa:CFP-NTR)* embryos at 54, 80, and 100 hpf (Figure 4-4A-B, Figure 4-15A-F). We noted a striking disorganization of islet cells in the *insaMO*-injected larvae. Characteristically,  $\alpha$  cells are located in the islet mantle and  $\beta$  cells are clustered in the islet core in both zebrafish and mouse islets (Figure 4-4A, Figure 4-15A-C). However, in *insaMO*-injected islets, this islet architecture was inverted: a core of glucagon<sup>+</sup>  $\alpha$  cells was surrounded by insulin<sup>+</sup>  $\beta$  cells (Figure 4-4B, Figure 4-15D-F). Furthermore, as in *dnIRS2* embryos, *insaMO*-injected islets contained a decreased number of  $\alpha$  cells (Figure 4-4I). Moreover, we found that this impaired level of  $\alpha$  cell development in the insulin knockdown in embryos was sustained into adulthood (Figure 4-15G-J). However, there was no significant influence on somatostatin<sup>+</sup> delta ( $\delta$ ) cell number. Also, the total endocrine cell number, as marked by *Tg(neurod:GFP)*, only slightly increased in 1 dpf *insaMO*-injected embryos. Together these data indicate that increased  $\beta$  cell content may come at the expense of  $\alpha$  cell fate in insulin-deficient islets (Figure 4-16).

To test this interpretation, we first examined the expression of Pdx1 in the  $\alpha$  cells of control and *insaMO*-injected islets. We found that in *insaMO*-injected islets Pdx1 expression was often aberrantly co-expressed in glucagon<sup>+</sup>  $\alpha$  cells (Figure 4-4C,D,J, and 4-17A-E), suggesting a failure of  $\alpha$  cell maturation or destabilization of  $\alpha$  cell fate.  $\alpha$  cells exhibit a degree of plasticity during development and regeneration. For instance, mis-expression of Pdx1 induces  $\alpha$  cell fate instability and results in  $\alpha$  to  $\beta$  transformation in some contexts (65). Furthermore, in zebrafish (271) and mouse (160,161) models of extreme  $\beta$  cell ablation,  $\alpha$  cells can spontaneously transdifferentiate into  $\beta$  cells through an intermediate Pdx1<sup>+</sup> glucagon<sup>+</sup> stage. Based on this, we hypothesized that the increase of Pdx1 expression in  $\alpha$  cells of *insaMO*-injected embryos would result in the instability of  $\alpha$  cell fate, particularly during  $\beta$  cell regeneration. To test this, we ablated  $\beta$  cells in *Tg(insa:CFP-NTR)<sup>s892</sup>* embryos with metronidazole (254,266), and then quantified *insa:CFP-NTR*<sup>+</sup>, glucagon<sup>+</sup>, and bihormonal *insa:CFP-NTR*<sup>+</sup> glucagon<sup>+</sup> cells in regenerating control and *insaMO*-injected islets. In *insaMO* knockdown islets we observed increased  $\beta$  cell regeneration, and that many of these regenerated  $\beta$  cells were co-labeled with glucagon (Figure 4-4E, F, K). To distinguish between the possibilities that

these double positive cells had formed by the differentiation of progenitor cells, or by the transdifferentiation of glucagon<sup>+</sup>  $\alpha$  cells, we performed genetic  $\alpha$  cell lineage tracing using the Hot-Cre system. Specifically, the *Tg(gcga:Cre)<sup>s962</sup>* line was used together with *Tg(hsp70l:loxp-mcherry-stop-loxp-H2B-GFP;cryaa:CFP)<sup>s923</sup>* to label  $\alpha$  cells with H2B-GFP via heat-shock before  $\beta$  cell ablation (271). Thus, if  $\alpha$  cell identity was lost during  $\beta$  cell regeneration, these pre-labeled  $\alpha$  cells would transform into glucagon<sup>-</sup> H2B-GFP<sup>+</sup> cells. Indeed, in *insaMO*-injected embryos we found that a significant population of  $\alpha$  cells pre-labeled in this fashion lost glucagon expression during  $\beta$  cell regeneration (Figure 4-17F-H), suggesting that some  $\alpha$  cells do not maintain their fate after *insulin* knockdown. The loss of glucagon expression could indicate that  $\alpha$  cells have de-differentiated, or have been converted into another endocrine cell type. However, we hypothesize that  $\alpha$  to  $\beta$  cell conversion predominates after loss of insulin signaling as observed in previously published studies of transdifferentiation after  $\beta$  cell ablation in zebrafish (271).

To further test this hypothesis we again used the LRC lineage tracing approach, in which all early differentiated endocrine cells (<40 hpf) of the principal islet are labeled because of their quiescence. In this experiment, cells of any endocrine subtype that transdifferentiate into  $\beta$  cells during regeneration were labeled with H2B-RFP, while those arising by neogenesis were unlabeled (271). Consistent with our interpretation of  $\alpha$  to  $\beta$  cell transdifferentiation, we found that the  $\beta$  cell ablated *insaMO*-injected islets showed a 2.5 fold increase of regenerated H2B-RFP<sup>+</sup>  $\beta$  cells, but no change in the quantity of regenerated H2B-RFP<sup>-</sup>  $\beta$  cells (Figure 4-4G, H, L). Together, these data suggest that loss of insulin signaling increases  $\beta$  cell regeneration mainly through the increase of  $\alpha$  cell conversion. In summary, we have demonstrated that blocking of insulin signaling via *insaMO* decreases  $\alpha$  cell formation and induces Pdx1 expression in  $\alpha$  cells. Furthermore, the increased  $\alpha$  to  $\beta$  cell conversation following cell ablation in *insaMO* embryos may result from instability of the  $\alpha$  cell fate consequent to diminished of insulin signaling.

**Loss of insulin signaling in transplanted blastomeres promotes  $\beta$  cell generation.**

To distinguish whether the precocious  $\beta$  cell differentiation observed in our insulin signaling-knockdown models was due to autonomous loss of insulin signaling in endodermal progenitors, we used mosaic analysis. For these, *sox32* mRNA was injected into donor zygotes to drive endoderm formation while *sox32* morpholino was used to block host embryo endoderm formation (328); in these chimeras, transplanted donor cells replaced most endodermal tissues in host embryos (Figure 4-5A-B). Thus, we generated chimeric embryos in which endodermal gene expression was independently manipulated. When we injected *dnIRS2-GFP* mRNA into donor zygotes to specifically block insulin signaling in the transplanted cells, we found that the differentiating donor endoderm showed an expanded Pdx1<sup>+</sup> domain, identical to that seen with global *dnIRS2* over expression (Figure 4-5C-D). This result indicates that the endodermal pancreas progenitor cells directly respond to insulin signaling by suppressing Pdx1 expression.

Thus far our findings have demonstrated that in a developmental context insulin signaling acts as a negative regulator, inhibiting the early differentiation of ventral pancreatic progenitors. This prompted the question of whether blocking insulin signaling in transplanted multipotent progenitor cells, such as stem cells, could favor their differentiation into  $\beta$  cells in a more mature context. To address this in a manner that would be relevant to stem cell therapy, we performed a novel blastula to larval cell transplantation assay (Figure 4-6A-C). For this, *sox32* mRNA-injected donor blastula endoderm cells were transplanted into larval stage hosts, adjacent to the pancreas. First, to validate this approach, we verified the survival and integration of donor endoderm into host tissues; we transplanted donor blastula cells from *Tg(ubi:zebrabow)* embryos that expressed a ubiquitous RFP label, into host *Tg(flk1:GFP)* larvae that expressed GFP throughout the vasculature. We found that blood vessels pervaded the transplanted donor tissue (Figure 4-6D-E), demonstrating an interaction between the donor and host tissues. At one day post transplantation we found that about 80% of host embryos (75/90) had successfully integrated transplanted donor cells.

We next analyzed the differentiation of these endoderm-committed blastomeres in the context of wild type or  $\beta$  cell-ablated hosts. First, we found that control donor cells differentiated into *sox17: GFP*<sup>+</sup> endodermal cells by 1 day post transplantation in wild type hosts, but did not further differentiate into Pdx1<sup>+</sup> cells (Figure 4-6I, Figure 4-18A).

However, when control donor cells were transplanted into  $\beta$ -cell ablated host larvae, some Pdx1<sup>+</sup> cells were detected in control donor tissue after 1 day post transplantation (Figure 4-6F, I, Figure 4-18B). This finding revealed that  $\beta$  cell ablation in hosts non-autonomously influences the differentiation of transplanted donor endoderm cells. Next, when insulin signaling in the transplanted donor cells was impaired by *dnIRS2* mRNA injection, we observed a significant increase of Pdx1 induction in donor cells after transplantation; this increase was even more striking in donor cells that were integrated into  $\beta$  cell ablated hosts (Figure 4-6H,I, Figure 4-19C). Together, these results indicate that insulin signaling in the endoderm-committed progenitor-like cells autonomously regulated Pdx1 expression, and that this was further influenced by  $\beta$  cells in a non-autonomous manner.

Next, to determine whether donor cells can differentiate into pancreatic endocrine cells, we examined insulin and glucagon protein expression two days after transplantation. No insulin or glucagon positive cells were detected in donor cells that were transplanted into non-ablated host larva (data not shown) and this is consistent with the absence of Pdx1 induction in a non-ablated host environment. In contrast, when donor cells were transplanted into  $\beta$  cell-ablated host larvae, they differentiated into both insulin<sup>+</sup>  $\beta$  cells and glucagon<sup>+</sup>  $\alpha$  cells (Figure 4-7A,B, Figure 4-19A,B). Furthermore, when donor cells that were injected with *dnIRS2* mRNA were transplanted into  $\beta$  cell ablated host larvae, there was a significant increase of insulin expression in the transplanted cells, while, no glucagon<sup>+</sup> cells were detected (Figure 4-7C-E, Figure 4-19C). This is in accord with our earlier results that showed that inhibition of insulin signaling impairs  $\alpha$  cell differentiation. Lastly, in a reciprocal manner, we found that the presence of transplanted *dnIRS2* endoderm, but not control endoderm, increased the competence of host tissue-derived pancreata for  $\beta$  cell regeneration (Figure 4-7F, Figure 4-20). In summary, here we have demonstrated that insulin signaling regulates Pdx1 expression and  $\beta$  cell formation in transplanted blastula stage endodermal progenitor cells. However, further differentiation toward pancreatic and  $\beta$  cell fates requires additional permissive signals secreted by the host larva environment in response to  $\beta$  cell ablation.



#### IV.D. Discussion

The coordinated growth and development of the pancreatic  $\beta$  cell mass involves many phases, including specification of the pancreatic progenitor population, its expansion,  $\beta$  cell differentiation, and their proliferation. Here, we provide evidence that insulin signaling acts as a negative feedback signal to regulate pancreatic progenitor cell differentiation. Using multiple approaches, we have shown that when progenitors lacking insulin signaling they are primed for early differentiation into  $\beta$  cells. As such, insufficient insulin signaling influences the compensatory production of  $\beta$  cells via neogenesis. This precocious  $\beta$  cell differentiation during embryonic development may have long-term negative effects by prematurely depleting the pancreatic progenitor pool and destabilizing  $\alpha$  cell fate. In addition, using a novel blastomere-to-larva transplantation strategy, we found that loss of insulin signaling in the endoderm committed blastomeres drove their differentiation into  $\beta$  cells. Taken together, our data suggest that modulation of insulin signaling will be crucial for  $\beta$  cell replacement strategies that hinge upon in vitro and in vivo approaches to  $\beta$  regeneration for the treatment of diabetes.

#### A conserved role for insulin signaling in islet development

In many vertebrate species, there are two waves of pancreatic endocrine cell differentiation during primary and secondary transition stages (226,277,323,329). The first wave appears shortly after the specification of pancreatic progenitor domain, persists during its expansion, preceding the main pancreas progenitor differentiation program (54,74,291,313,330). Our results suggest are consistent with a role for insulin as a local, physiologically relevant signal that feeds back to block pancreas progenitor cell differentiation, precisely regulating their expansion. Loss of insulin signaling initially induces compensatory differentiation of progenitor cells into  $\beta$  cells. Consistent with this, a comparable response was also observed in mice lacking *insulin* expression. Although loss of both mouse *Insulin* orthologues is required to induce hyperglycemia, islet hyperplasia was observed in the single *Ins1* and *Ins2* knockouts, and double null mice (320). Indeed, islet size is significantly increased in *Ins1<sup>-/-</sup>Ins2<sup>-/-</sup>* mice at birth and the  $\beta$  cell mass in *Ins2<sup>-/-</sup>* mice was increased almost threefold at 7 weeks of age, indicating that

increased  $\beta$  cell mass expands to compensate for low insulin production (320,324). This is further supported by the finding that expression of human *INSULIN* in the *Ins1*<sup>-/-</sup>; *Ins2*<sup>-/-</sup> mouse reverses the compensatory  $\beta$  cell hyperplasia (331). These findings together with our results support the hypothesis that insulin acts as a negative regulator for  $\beta$  cell formation during development (320,324). However, this hypothesis appears to be contradicted by findings in *Insr*<sup>-/-</sup> mice. These mice initially appear unaffected as neonates, showing a normal  $\beta$  cell mass; however, immediately after feeding, hyperglycemia and hyperinsulinemia develop due to severe insulin resistance, and this results in neonatal death (317,318). This contradictory result may be due to compensatory/redundant activation of IGF-1 receptors in the absence of Insulin receptors (319). It has been proposed that the local production of IGF-1 in the pancreas acts as a negative regulator factor of  $\beta$  cell mass, as knockout of *Igf1* in pancreas increases  $\beta$  cell differentiation and a 2.3 fold enlarged islet cell mass (332). Thus, the de-repression of  $\beta$  cell differentiation resulting from loss of InsR signaling is likely countered by redundant activation of IGF1R signaling, explaining the contradictory results. On the other hand, our data does not exclude the possibility that insulin regulates pancreatic progenitor differentiation in part via IGF1R, given the complex interaction between the insulin and IGF1 signaling pathways (310,319). However, given that Insulin binds the IGF1R and InsR/IGF1R heterodimers with much lower affinity than InsR (333), it is likely that our manipulations affect insulin receptor signaling rather than IGF1R signaling. In further support of this interpretation, knockdown either of *Igf1* or *Igf1R* in zebrafish results in severe neural and gross developmental defects (334,335), which differ significantly from the *insulin* knockdown phenotypes reported here. This supports our conclusion that insulin receptor signaling is the major insulin family member that suppressing differentiation of pancreas progenitor cells.

### **Insulin signaling maintains pluripotency in multiple contexts**

Insulin secreted by pancreatic  $\beta$  cells is one of the most important nutrient sensors. It has been classically viewed as a mitogen, stimulating proliferation and promoting cell survival (310). However, recent studies have shown that insulin plays key roles both in

maintaining stem cell fate and regulating stem cell differentiation (146-148,336). For example, self-renewal of human embryonic stem (ES) cells requires the activation of insulin and IGF signal pathways; blocking these signals promotes ES cell differentiation (146,336). Also, in *Drosophila larvae*, starvation reduces the number of hematopoietic progenitor cells by increasing their differentiation, and these starvation effects are mediated by the insulin signaling pathway (148,337). Furthermore, reduced systemic insulin signaling and down-regulation of the downstream insulin signaling component Akt/PI3K promotes *hematopoietic and skin stem* cell differentiation (148,338).

Consistent with previous findings, our study clearly supports a role for insulin in regulating progenitor cell differentiation in the developing pancreas, which is reminiscent to the well-described roles of Notch in controlling pancreatic progenitor cell differentiation. Notch signaling in pancreatic progenitors stimulates self-renewal in this progenitor pool and blocks endocrine cell differentiation via repression of the bHLH transcription factor Ngn3 (110,339). Moreover, Notch signaling is required to sustain Ptf1a expression in early pancreatic progenitors (340). Disruptions of Notch signaling in early pancreatic progenitor cells induces premature differentiation of pancreatic endocrine cells and diminishes the pancreatic progenitor pool (110,341). The strong phenotypic similarities of insulin signaling and Notch signaling loss suggest that the pathways interact: insulin signal may act as a positive regulator of Notch signaling in pancreatic progenitor cells. Indeed, there is accumulating evidence supporting intensive cross talk between PI3K/Akt and Notch pathways. For instance, during megakaryocyte development the PI3K/AKT pathway is activated by Notch stimulation, which in turn enhances Notch-dependent differentiation (342). Cooperation between Notch and insulin signaling may therefore integrate environmental and metabolic cues to regulate progenitor cell maintenance and differentiation. Therefore, we hypothesize that, inhibition of insulin signaling in pancreatic progenitor cells would impair positive circuits between PI3K/Akt and Notch, activating the endocrine differentiation program, and resulting in the premature differentiation of pancreatic progenitor into  $\beta$  cells. Additional studies in our lab are aimed at further clarifying the interaction of notch and insulin signaling during progenitor differentiation.

## Potential mechanisms of insulin signaling in endocrine cell fate regulation

In addition to regulating pancreatic progenitor cell differentiation, our data also suggest that insulin plays a crucial role in endocrine subtype specification during differentiation. After blockade of insulin signaling we found an increase of  $\beta$  cell formation from the pancreatic progenitors and a decrease of  $\alpha$  cell fate. This function of insulin signaling may be partially mediated via regulation of Pdx1 expression as our results show that loss of insulin signaling promotes Pdx1 expression in pancreas progenitors as well as transplanted blastula stem cells. As one of the key transcription factors in regulating  $\beta$  cell specification and function, Pdx1 enhances transcription of  $\beta$  cell programmed genes such as *Insulin*, *Glut2*, *Glucokinase*, and *Mafa* but inhibits  $\alpha$  cell programmed genes like *Arx* and *Glucagon* (343-345). Enforcing expression of Pdx1 in pancreatic endocrine progenitor cells result in increased  $\beta$  cell number and decreased  $\alpha$  cell number during embryonic stages (65). Besides regulating pancreatic progenitor differentiation, Pdx1 has also been shown by previous studies to be able to directly drive  $\alpha$  to  $\beta$  cell fate switch. In mouse models, mis-expressing Pdx1 in  $\alpha$  cells drove postnatal conversion of glucagon<sup>+</sup> Pdx1<sup>+</sup> cells into  $\beta$  cells (65). Moreover, we have recently shown that in zebrafish models, these Pdx1 mis-expressing immature “ $\alpha$ -like” cells are unstable, and can transdifferentiate into  $\beta$  cells (271). Our studies have indicated in addition to loss of  $\alpha$  cell number, loss of insulin during development induced the formation of Pdx1<sup>+</sup> glucagon<sup>+</sup> “ $\alpha$  like” cells. These “ $\alpha$ -like” cells appeared to be unstable and therefore they are likely to be the source of increased  $\alpha$  to  $\beta$  cell conversion after insulin loss.

The induction of Pdx1 in response to diminished insulin signaling may be due to activation of the forkhead transcription factor Foxa2, which is negatively regulated by insulin signaling (346-348). Foxa2 binds to the Pdx1 promoter region and is crucial for Pdx1 expression in pancreatic progenitors and mature  $\beta$  cells (343,349,350). Furthermore, it has been proposed that activation of both insulin and Notch represses Foxa2 activity (346,348,351-353). Thus, we propose a model in which reduced insulin and Notch signaling activities enhances the transcription and nuclear translocation of Foxa2 in endodermal progenitor cells; together this drives the activation of Pdx1.

The forkhead transcription factor Foxo1 also has important roles in metabolic

regulation downstream from insulin signaling (354,355). The loss of *Foxo1* results in  $\beta$  cell dedifferentiation, revealing a role in  $\beta$  cell fate maintenance (39). Similarly, it is likely that Foxo1 also has a hand in insulin-regulated pancreatic progenitor differentiation. In mature  $\beta$  cells, Foxo1 and Pdx1 exhibit mutually exclusive localization in the nucleus and Foxo1 blocks Pdx1 transcription by disrupting Foxa2-dependent transcription at the Pdx1 promoter (356). Thus insulin signaling may be regulating the expression of Pdx1 via multiple mechanisms, including forkhead proteins and Notch, and ultimately it is the inappropriate expression of Pdx1 that drives  $\beta$  cell hyperplasia and  $\alpha$  cell hypoplasia in our insulin knockdown models. This hypothesis, that Foxa2 and Foxo1 are the link between insulin signaling and Pdx1 expression, is under investigation in our lab; future studies are aimed at clarifying this and other downstream mechanisms of insulin during regulation of pancreas development.

### **A potential role for insulin signaling in programming $\beta$ cells *in vitro***

Understanding and manipulating the regulatory mechanisms of insulin signaling in pancreatic progenitors during developmental stages can potentially facilitate the programming of embryonic stem cells into  $\beta$  cells *in vitro*. When we transplanted endoderm-fated blastomeres, which are similar to embryonic stem cells, into zebrafish larvae, the dnIRS2-expressing “insulin resistant” blastomeres differentiated into Pdx1<sup>+</sup> progenitor cells and  $\beta$  cells with a much greater efficiency than wild type controls. This result indicates that blocking this feedback action of insulin signaling in endoderm progenitors may facilitate  $\beta$  cell production *in vitro*. However, we also observed that the transplanted blastomeres only expressed the  $\beta$  cell markers Pdx1 and insulin when they were transplanted into  $\beta$  cell-ablated host larvae. This result demonstrates that blocking insulin signaling alone is not sufficient to drive  $\beta$  cell production and that additional signaling factors released from host larvae in response to  $\beta$  cell ablation are needed to facilitate  $\beta$  cell differentiation. Furthermore, our results reveal bidirectional signaling, as these “insulin resistant” blastomeres can also indirectly promote  $\beta$  cell regeneration in host tissues. This is consistent with previous studies that show that transplanted stem cells can secrete angiogenic and growth factors (357,358). In fact, some functional benefits

observed after stem cell transfer might be due in part to the secretion of soluble factors that act as paracrine or endocrine fashion to promote tissue regeneration (357,359,360). Thus, we propose that in our model, the pancreatic progenitors interact through the release of secreted signaling factors that act as paracrine or systemic signals. Additional studies are needed to identify these signaling factors and to test this hypothesis; unveiling these mechanisms will be essential for understanding the deficiencies of  $\beta$  cell compensation that are seen in insulin resistant and diabetic states.

Lastly, recent studies have indicated that endogenous pancreatic multipotent progenitor (PMP) cells are present in adult pancreas both in human (361-363) and mouse (176,364), and that metabolic stress can influence their proliferation and differentiation into  $\beta$  cells (364). Maintaining the balance of self-renewal and differentiation in these progenitor cells is likely to be crucial for long-term  $\beta$  cell compensation in response to metabolic stresses. Given our findings that insulin signaling plays an important role in regulating the progenitor cell differentiation during development, understanding the roles and mechanisms of insulin signaling in adult pancreatic progenitors, and how to appropriately manipulate insulin signaling in these populations, may prove to be critical for developing new regenerative therapies for diabetes.

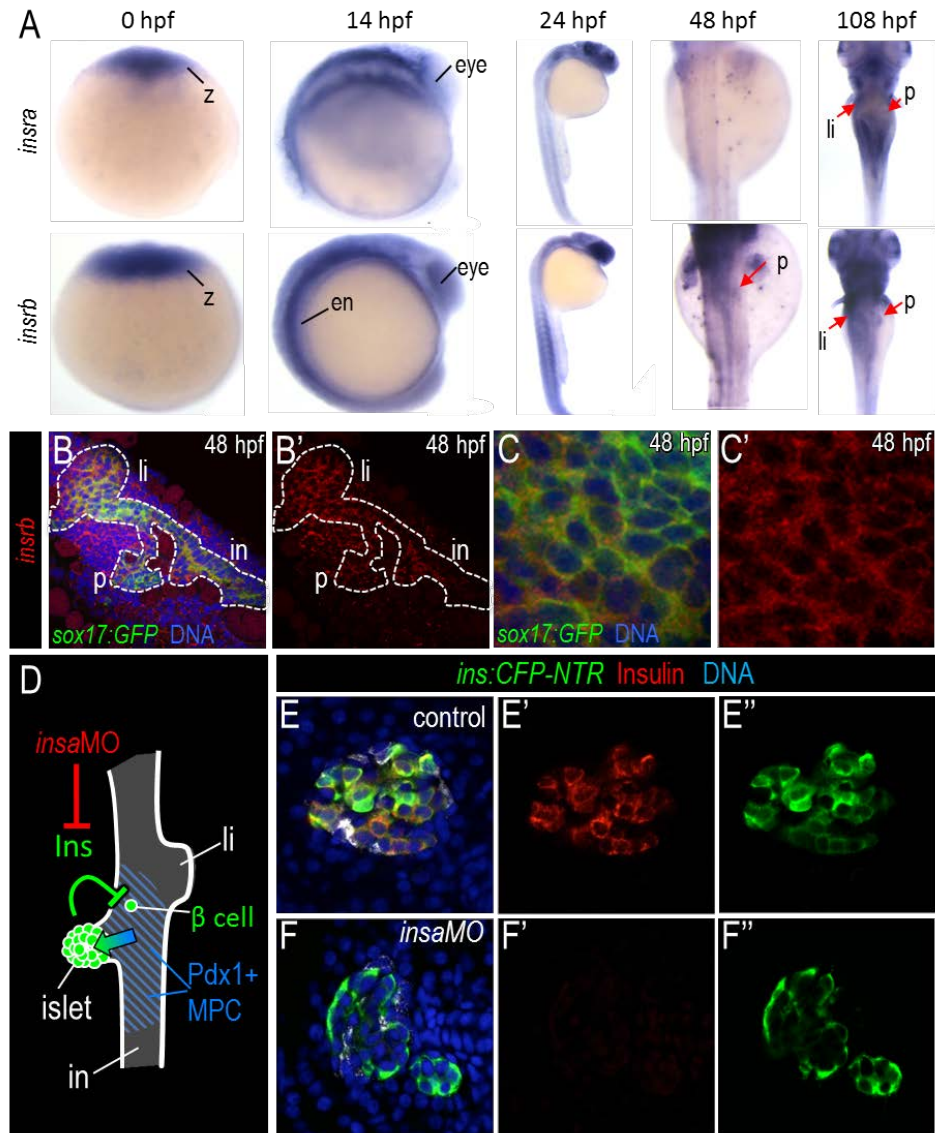


Figure 4-1. Inhibition of insulin signaling in pancreatic progenitor cells via *insaMO*. (A) In situ hybridization for *insra* and *insrb* in the developing zebrafish embryos. (B-C) Fluorescent in situ hybridization of *insrb* (red) in *Tg(sox17:GFP)* endoderm (green) at 48 hpf. (D) Schematic of blocking endoderm receiving insulin signaling from dorsal  $\beta$  cells via *insulin* morpholino (*insaMO*) knockdown. (E-F) Confocal plane of 3 dpf *Tg(insa:CFP-NTR)* control and *insaMO* injected islets stained for CFP (green), insulin (red) and glucagon (white). Abbreviations: z, zygote; ed, endoderm; p, pancreas; li, liver.

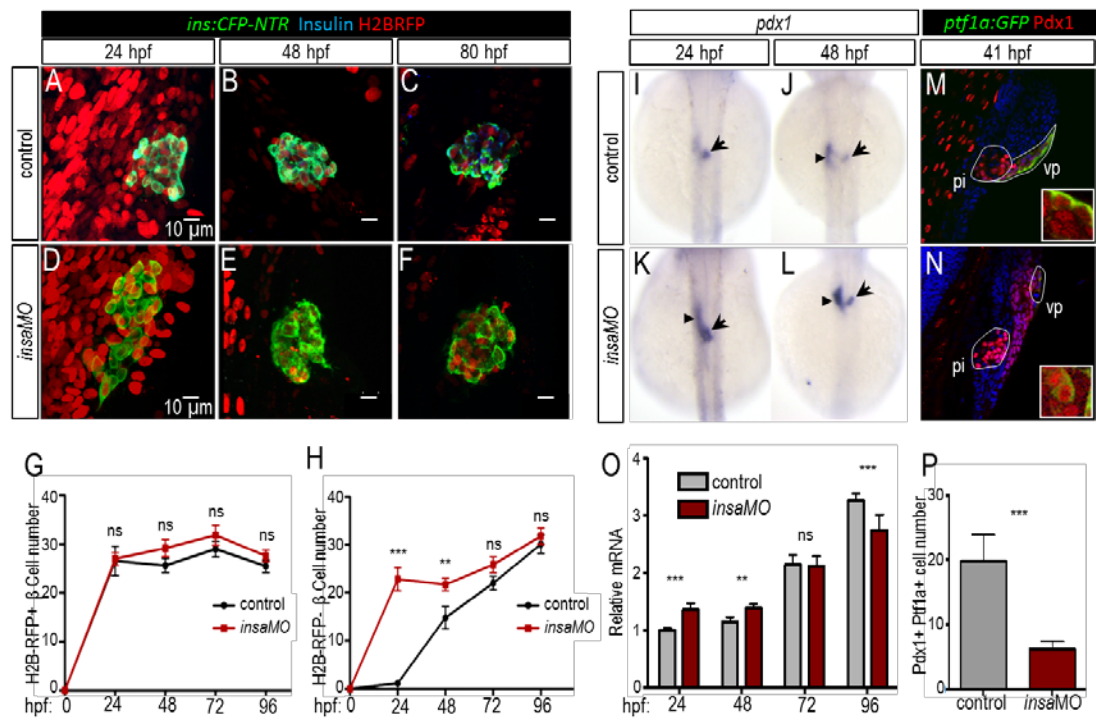




Figure 4-2. *insulin* knockdown increases Pdx1 expression and drives early pancreatic progenitor differentiation into  $\beta$  cells. (A-F) Confocal planes of 24 hpf, 48 hpf, and 80 hpf *Tg(insa:CFP-NTR)* control (A-C) and *insaMO* (D-F) islets stained for CFP (green) and insulin (blue). H2B-RFP mRNA was zygotically-injected to distinguish dorsal (H2B-RFP<sup>+</sup>) and ventral (H2B-RFP<sup>-</sup>) pancreas derived  $\beta$  cells. (G) Quantification of H2B-RFP<sup>+</sup>  $\beta$  cell number in control and *insaMO*-injected islets from 24 hpf to 96 hpf. (H) Quantification of H2B-RFP<sup>-</sup>  $\beta$  cell number in control and *insaMO* injected islets from 24 hpf to 96 hpf. (I-L) In situ hybridization of *pdx1* expression in 24 hpf and 48 hpf control (I, J) and *insaMO* injected embryos (K, L). Black arrows show the pancreatic principal islet. Black arrow heads indicate the expression of Pdx1 in ventral endoderm. (M-N) Confocal plane of 48 hpf *Tg(ptf1a:GFP)* control and *insaMO* injected endoderm stained for CFP (green) and Pdx1(red). pi: principal islet. (O) Real time PCR to detect Pdx1 expression in both control and *insaMO* injected embryos from 24 hpf to 96 hpf. (P) Quantification of the number of Pdx1<sup>+</sup>Ptf1a<sup>+</sup> pancreatic progenitor cells in both control and *insaMO* injected embryos. One way ANOVA was used in O and Student's t-test was used in P for the statistical analyses.

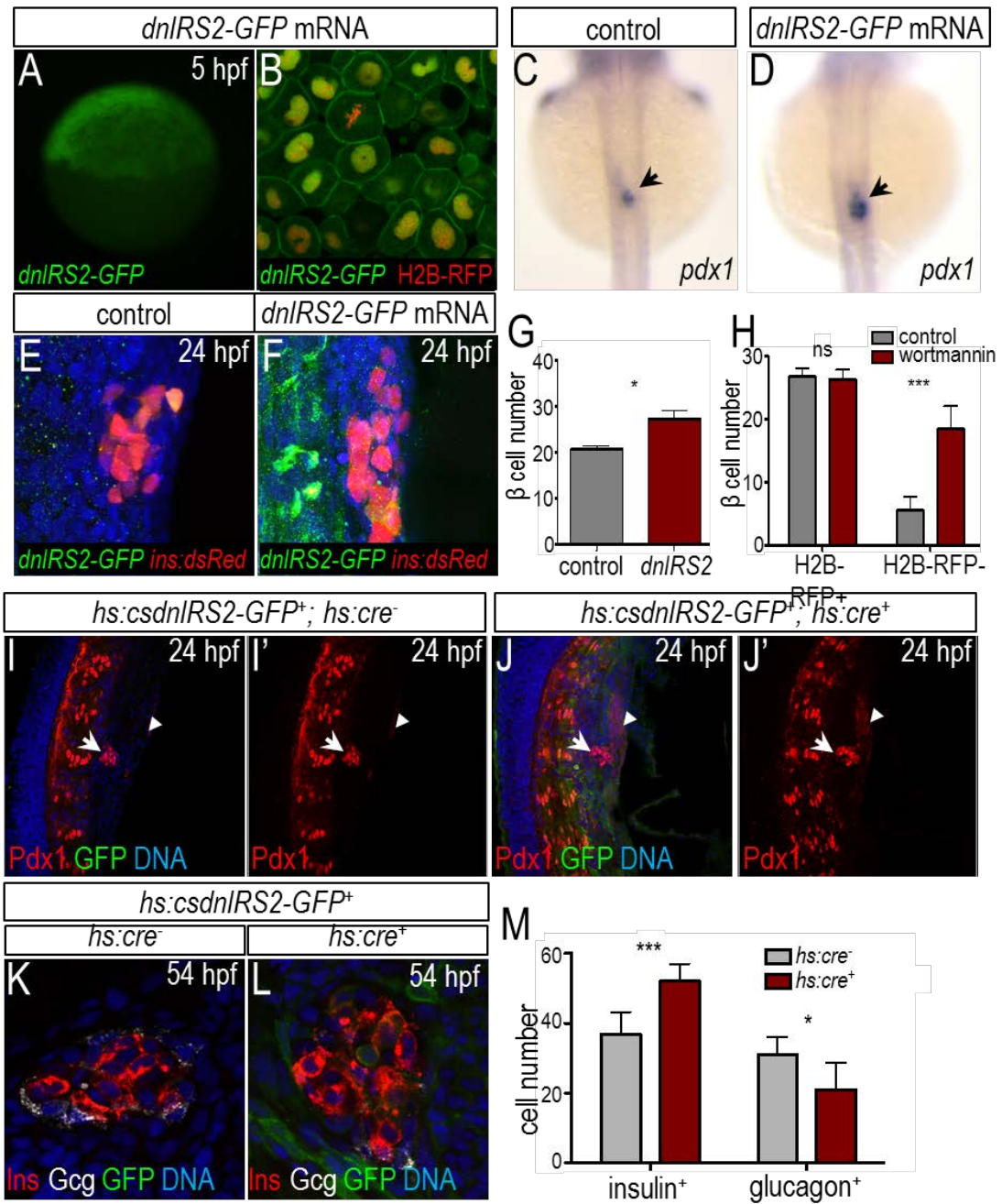


Figure 4-3. Insulin signaling blockade drives early differentiation of ventral pancreatic progenitors. (A) 5 hpf embryo injected with *dnIRS2-GFP* mRNA shows global green fluorescence. (B) Confocal plane shows the localization of dnIRS2-GFP to the plasma membrane. Co-injected H2B-RFP mRNA labels cell nuclei. (C-D) In situ hybridizations show *pdx1* expression in control (C) and dnIRS2-GFP mRNA-injected (D) embryos. (E-F) Confocal projections of 1 dpf *Tg(insa:dsRed)* islets in control (E) and *dnIRS2-GFP* mRNA-injected (F) embryos. (G) Quantification of *insa:dsRed*<sup>+</sup>  $\beta$  cells in 24 hpf control and *dnIRS2-GFP*mRNA injected embryos. (H) Quantification of  $\beta$  cells derived from dorsal pancreas (H2B-RFP<sup>+</sup>) and ventral pancreas (H2B-RFP<sup>-</sup>) in 30 hpf control DMSO-treated and 1  $\mu$ M wortmannin treated embryos. (I-J) Confocal projection of 24 hpf *Tg(hs:CSdnIRS2-GFP)* (control) and *Tg(hs:csdnIRS2-GFP);Tg(hs:cre)* (*dnIRS2* mis-expressing) pancreatic endoderm stained for GFP (green) and Pdx1(red). White arrows indicate the principal islet and arrowheads indicate the ventral endodermal region. (K-L) confocal planes of 54 hpf *Tg(hs:CSdnIRS2-GFP)* and *Tg(hs:csdnIRS2-GFP);Tg(hs:cre)* islets stained for GFP (green), Insulin (red), and Glucagon (white). (M) Quantification of Insulin<sup>+</sup>  $\beta$  cells and Glucagon<sup>+</sup>  $\alpha$  cells in control (gray) and *dnIRS2* mis-expressing (red) groups. Student t-test was used in G and Two way ANOVA was used in H and M.

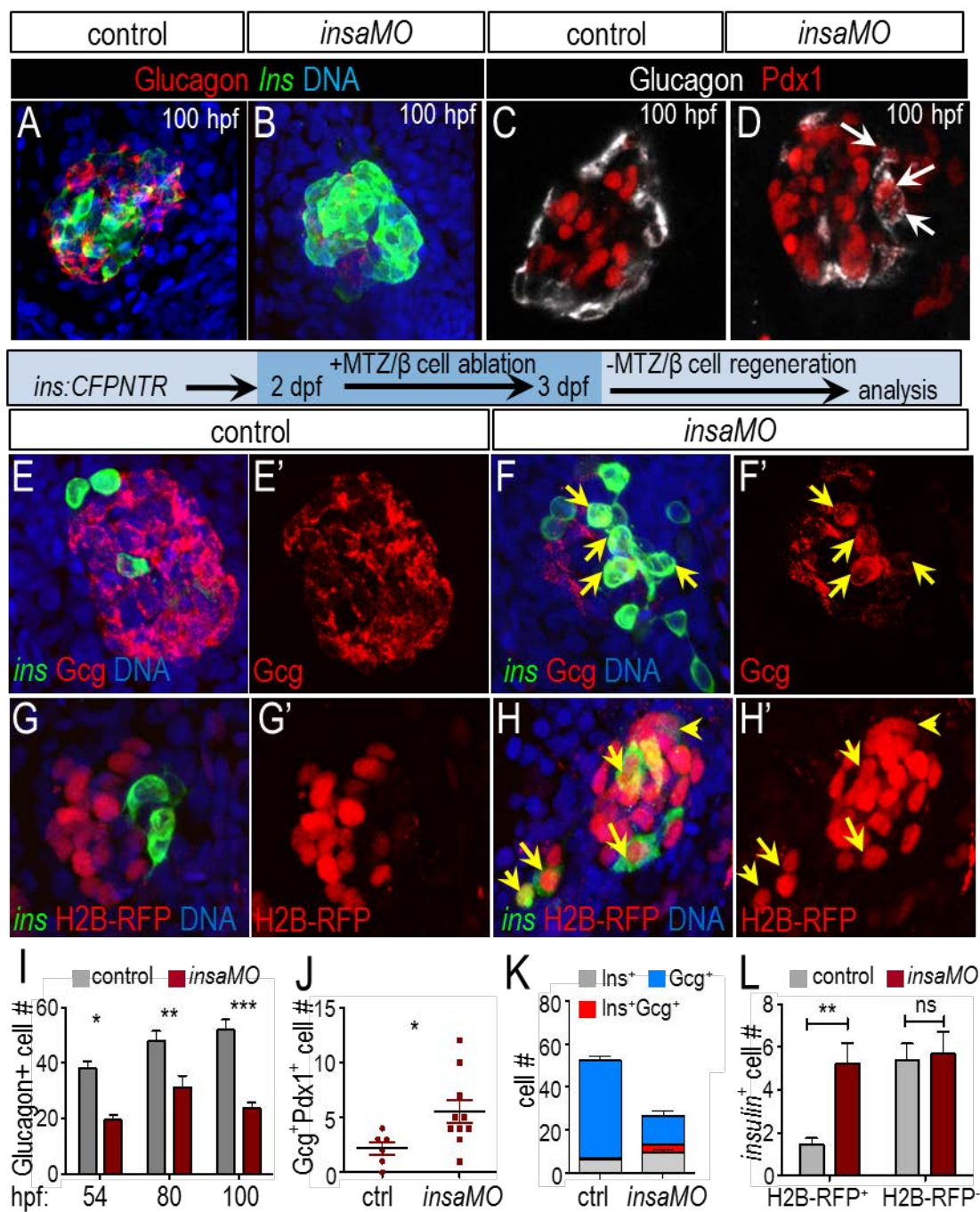


Figure 4-4. Insulin knockdown impairs  $\alpha$  cell development and destabilizes  $\alpha$  cell fate. (A-D) Confocal projections of 100 hpf *Tg(insa:CFP-NTR)* control (A,C) and *insaMO*-injected (B,D) islets stained for Glucagon (red) and CFP (green) (A,B), or Glucagon (white) and Pdx1 (red) (C,D). White arrows indicate Glucagon<sup>+</sup> Pdx1<sup>+</sup> double positive cells. (E-F) Confocal projections of 4 dpf *Tg(insa:CFP-NTR)* control and *insaMO*-injected regenerating islets that were stained for Glucagon (red) and CFP (green). Yellow arrows indicate *ins*<sup>+</sup> Glucagon<sup>+</sup> double positive regenerating  $\beta$  cells. (G-H) Confocal projections of 4 dpf *Tg(insa:CFP-NTR)* control and *insaMO*-injected regenerating islets labeled with H2B-RFP. Yellow arrows indicate H2B-RFP<sup>+</sup> *ins*<sup>+</sup> double positive regenerating  $\beta$  cells. (I) Quantification of Glucagon<sup>+</sup> cell number in 54 hpf, 80 hpf and 100 hpf control and *insaMO*-injected islets. (J) Quantification of Glucagon<sup>+</sup> Pdx1<sup>+</sup> double positive cells in 100 hpf control and *insaMO*-injected islets. (K) Quantification of *insa*<sup>+</sup>, Gcg<sup>+</sup>, and *insa*<sup>+</sup> Gcg<sup>+</sup> cells in 1 day post ablation (dpa) control and *insaMO*-injected islets. (L) Quantification of H2B-RFP<sup>+</sup> *insa*<sup>+</sup> and H2B-RFP<sup>-</sup> *insa*<sup>+</sup> regenerating  $\beta$  cells in 1dpa control and *insaMO*-injected islets. Two way ANOVA was used in I, K and Student's t-test was used in J.

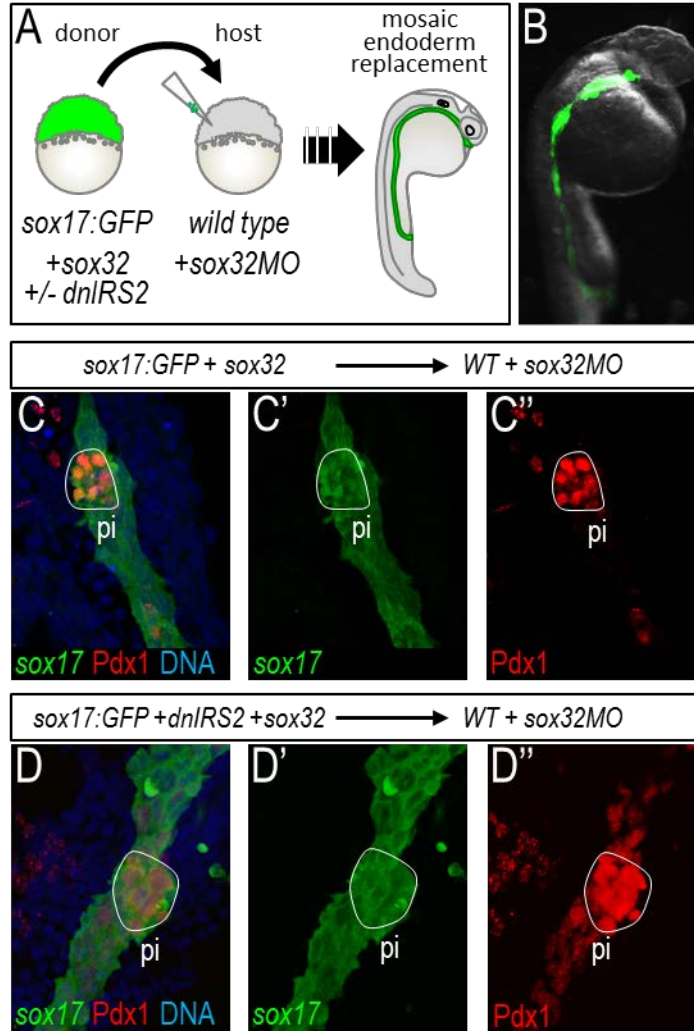


Figure 4-5. Insulin signaling blockade in transplanted endodermal progenitor cells promotes Pdx1 expression. (A) Endodermal transplantation scheme: blastula stage *Tg(sox17:GFP)* donor cells were transplanted into wild type host blastulae. (B) 1 dpf host embryo shows endoderm replacement with transplanted with *Tg(sox17:GFP)* donor cells. (C,D) Confocal projections of 1 dpf embryos with endoderm transplants from *sox32* mRNA (C; *n*=8) or *sox32* and *dnIRS2* mRNA (D; *n*=7) injected donors. Embryos were stained for GFP (green), Pdx1 (red), and DNA (blue).



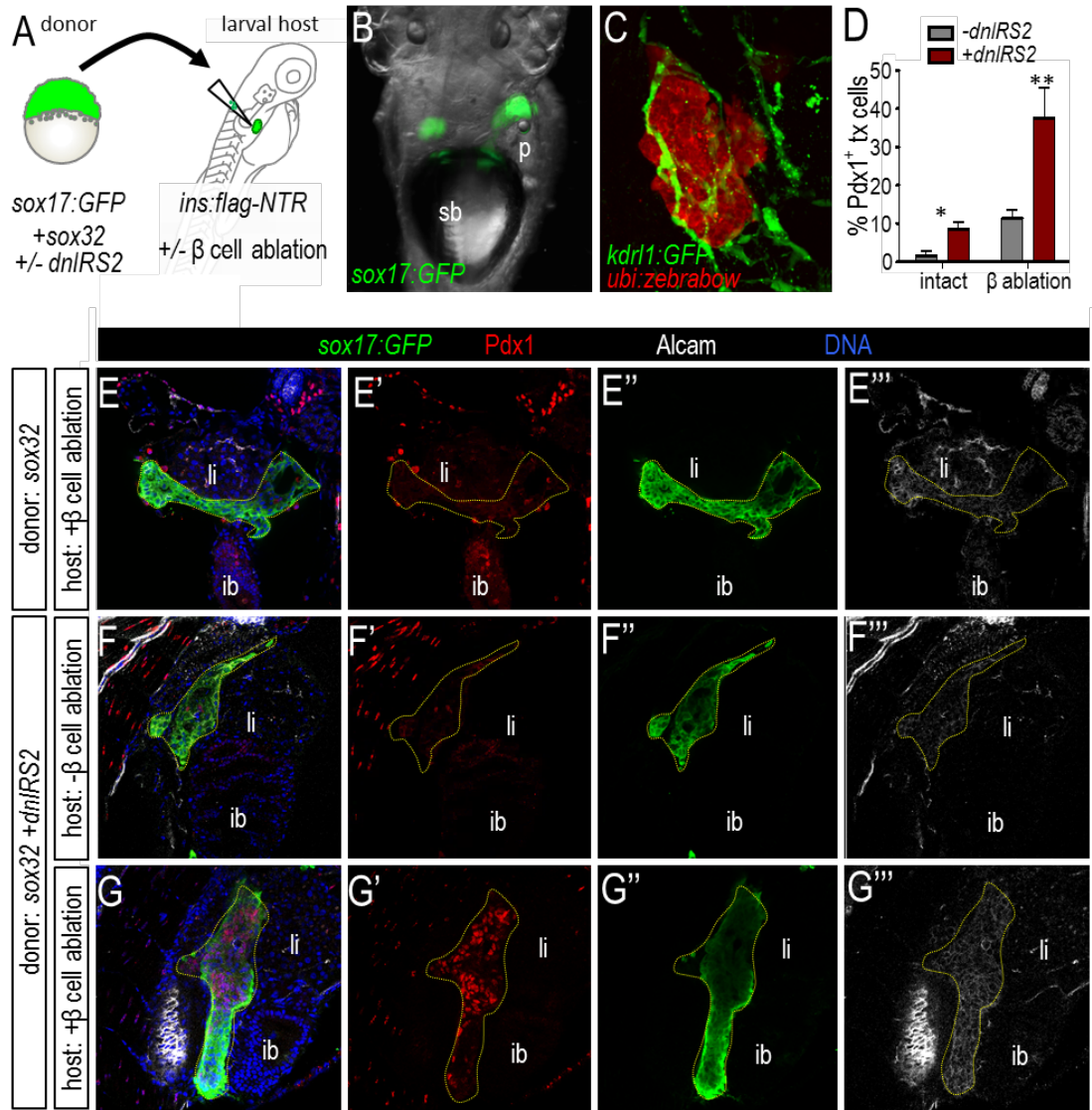


Figure 4-6. Insulin signaling blockade in transplanted endodermal progenitor cells increases Pdx1 expression after transplantation into  $\beta$ -cell ablated host larvae. (A) Blastula to 4 dpf larva cell transplantation scheme. (B) 5 dpf host larva shows transplanted *Tg(sox17:GFP)* donor cells at 1 day post transplantation. (C) Confocal projection of *Tg(flk1:GFP)* (green) host larva transplanted with *Tg(ubi:zebrabow)* donor cells (red) at 1 day post transplantation shows successful engraftment. (D) Quantification of percentage of Pdx1<sup>+</sup> cells in transplanted *sox17:GFP*<sup>+</sup> donor tissues. (E-G''') Merged and single channel confocal planes of chimeric host larvae show transplanted *Tg(sox17:GFP)* donor cells (outlined) at 1 day post transplantation, stained for GFP (green), Pdx1 (red), Alcam (white), and DNA (blue). (E-E''') *sox32* mRNA-injected blastomeres transplanted into  $\beta$  cell-ablated host larvae. (F-F''') *sox32* and *dnIRS2* mRNA-injected blastomeres transplanted into  $\beta$  cell-intact host larvae. (G-G''') *sox32* and *dnIRS2* mRNA-injected blastomeres transplanted into  $\beta$  cell-ablated host larvae. Student t-test was used for statistical analysis in D. Abbreviations: sb, swim bladder; p, pancreas; ib, intestine bulb; li, liver.



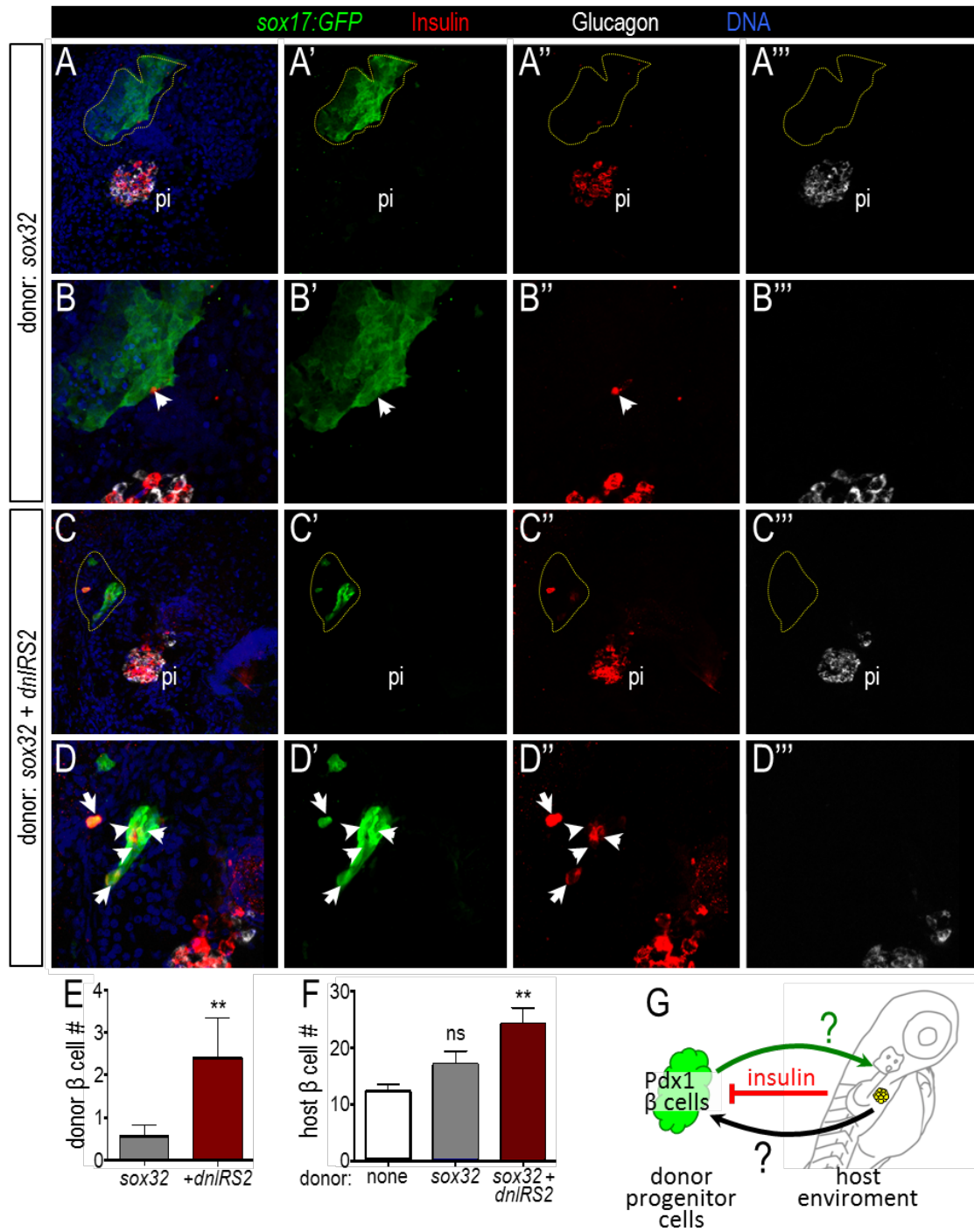


Figure 4-7. Insulin signaling blockade in donor cells promotes Insulin expression and host  $\beta$  cell regeneration. (A-D) Merged and single channel confocal projections of transplanted *Tg(sox17:GFP)* donor cells in  $\beta$  cell-ablated host larvae stained for GFP (green), Insulin (red) and Glucagon (white) at 2 days post transplantation. (A,B) donor cells injected with *sox32* mRNA only (C-D) donor cells were injected with *sox32* and *dnIRS2* mRNAs (E) Quantification of Insulin+  $\beta$  cells in donor tissues that were transplanted into  $\beta$  cell ablated hosts. (F) Quantification of host-derived regenerated  $\beta$  cells after transplantation with no cells (sham), or donor cells injected with *sox32* mRNA alone, or *sox32* plus *dnIRS2* mRNAs. Student's t-test was used in F and one way ANOVA was used in G. (G) Model for the role of insulin signaling in regulating  $\beta$  cell differentiation and interaction between transplanted endoderm progenitor cells and the host environment. Abbreviation: pi, host larvae principal islet at 2 day post  $\beta$  cell ablation.

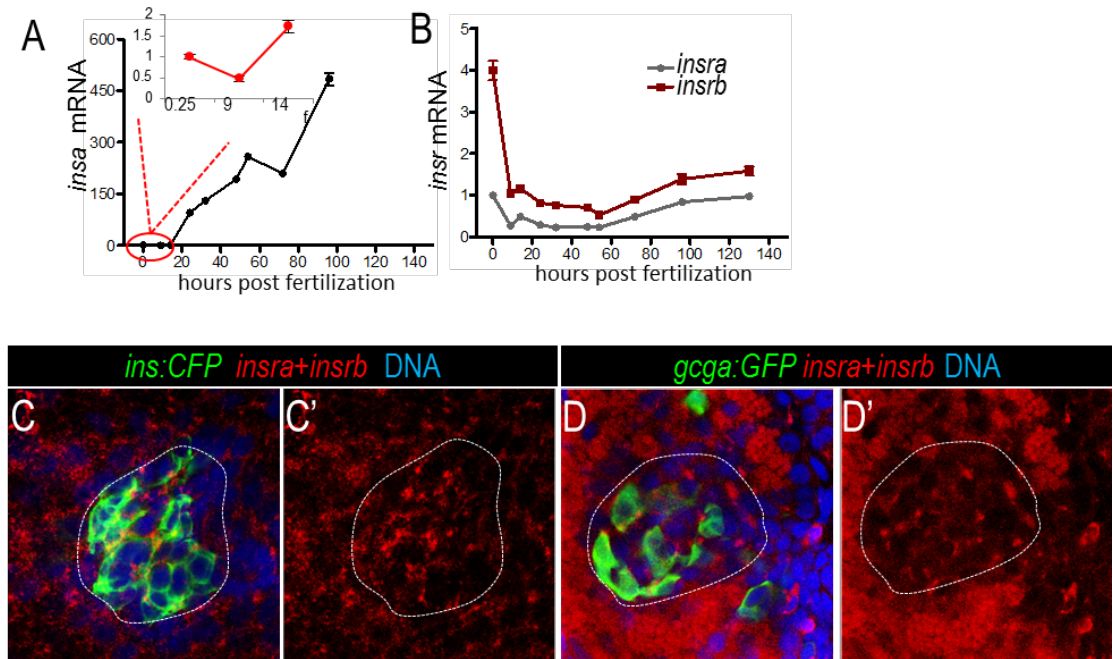


Figure 4-8. Expression of *insulin receptor* in pancreas during development. (A-B) real-time PCR analysis of *insulin*, *insulin receptor a* (*insra*) and *insulin receptor b* (*insrb*) in whole embryos at specific time points indicated in the paragraph.  $n=3$  for each time point. (C-D) fluorescent *in situ* hybridization indicating the expression of insulin receptors (red) in pancreatic islet  $\beta$  (*ins:CFP* in C, green) and  $\alpha$  cells (*gcg:GFP* in D, green). The *in situ* probe (*insra+b*) was designed to target a homologous region for both *insra* and *insrb* as indicated (Toyoshima, Y. et al., Endocrinology, 2008).

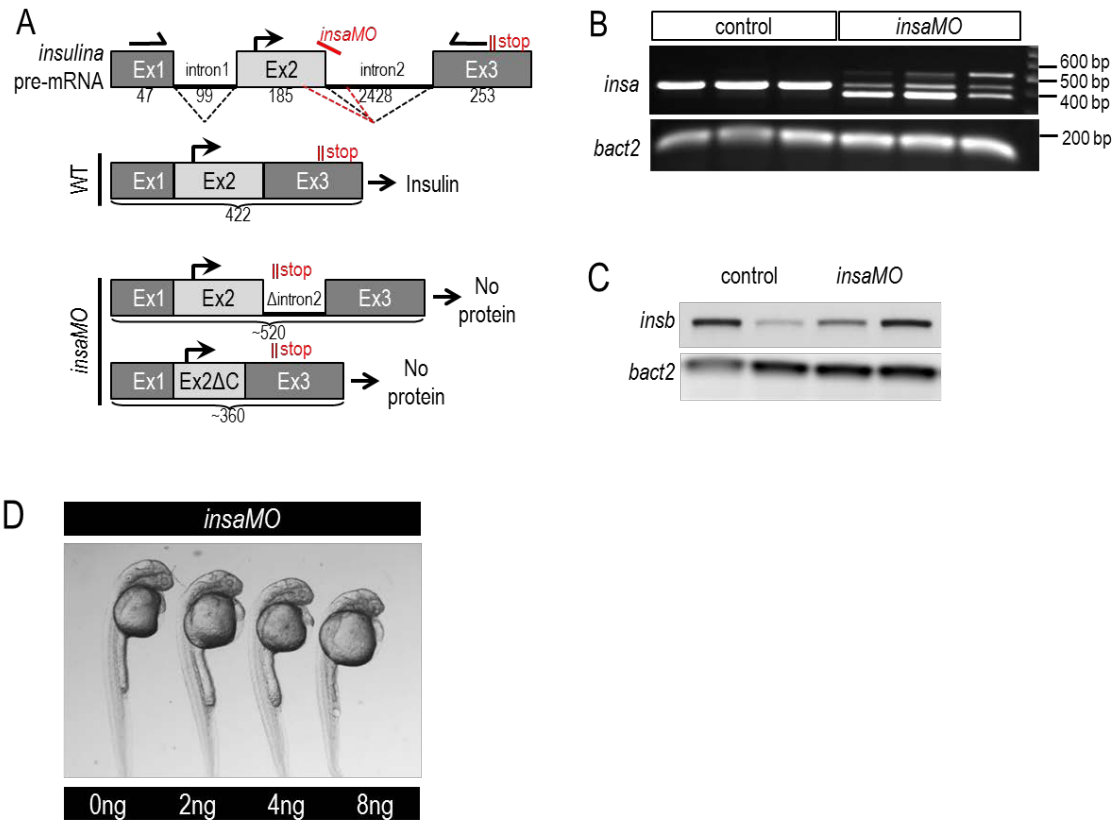


Figure 4-9. Knockdown of insulin via *insulin* morpholino (*insaMO*). (A) Diagram illustrating how *insaMO* interrupts the normal splicing of pre-insulin mRNA. *insaMO* is designed to target the exon2-intron2 boundary of *preproinsulin-a* mRNA. *insaMO* results in two alternative splicing mRNA products. The primer set was designed at exon 1 and exon 3 to amplify the mRNA products. (B) Agarose gel showing amplified *insulin* PCR products using mRNA extracted from 2 dpf control and *insaMO*-injected embryos. Note that a single PCR product with size of 420 bp was detected in control embryos but two aberrant PCR products with the sizes of 520 and 360 bp were detected in *insaMO*-injected embryos.  $\beta$  actin was used as internal control. (C) Expression of *insulin-b* mRNA in control and *insaMO*-injected embryos showed no alternation after *insaMO* injection. (D) No overt phenotype was observed in control embryos or those injected with increasing doses of *insaMO* from 2 ng to 8 ng.



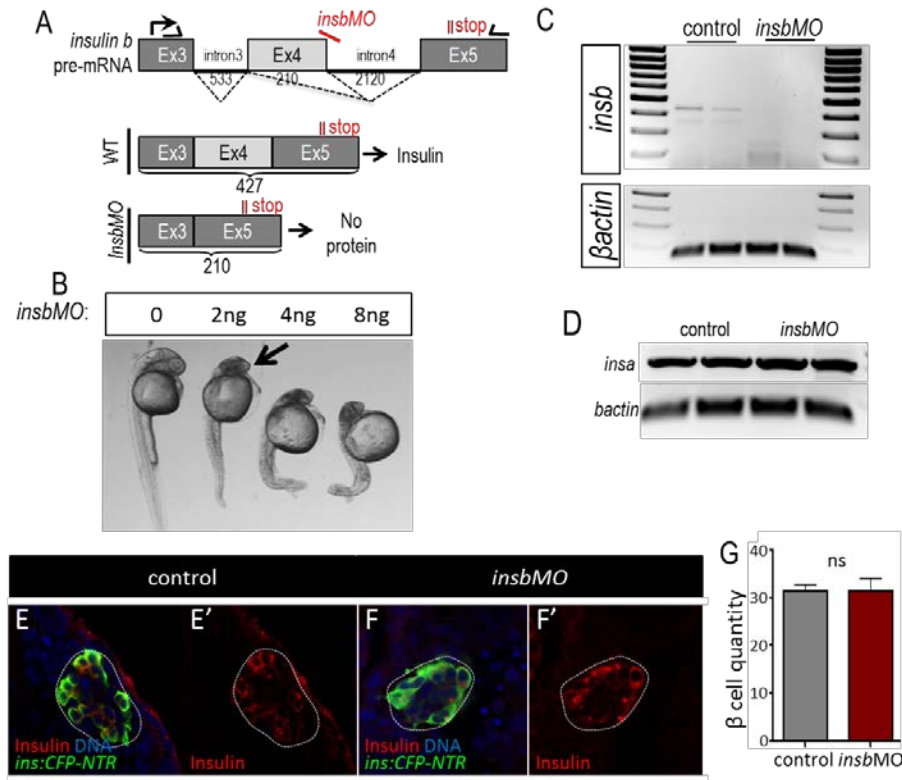


Figure 4-11. Knockdown of *insb* results in severe development defects. (A) Diagram illustrating how *insbMO* interferes with the splicing of *pre-insulin b* mRNA. *insbMO* binds the junction between exon 4 and intron 4 and induces the deletion of exon4. The primer set was designed to amplify between exon 3 and exon 5. (B) Dose dependent developmental defects at 48 hpf after *insbMO* injection. Arrow indicates the strong influence on head development using 2 ng *insbMO* injection. (C) Agarose gel showing amplified *insb* PCR product from 2 dpf control and *insbMO*-injected embryos. Note that PCR product with size of 427 bp was detected in control embryos (arrow head) but not *insbMO* injected embryos. A weak product of 210 bp can be detected in *insbMO*-injected embryos (arrow).  $\beta$  actin was used as internal control. (D) Expression of *insulin-a* mRNA in both control and *insbMO* injected embryos showed that knockdown of *insb* did not interrupt *insulin-a* mRNA transcription. (E-F) Confocal images of 24 hpf control and *insbMO* islets of *Tg(ins:CFP-NTR)* embryos stained for CFP (green) and insulin (red). (G) Quantification of *ins:CFP-NTR*<sup>+</sup>  $\beta$  cells in 24 hpf control(n=5) and *insbMO*-injected embryos(n=6).



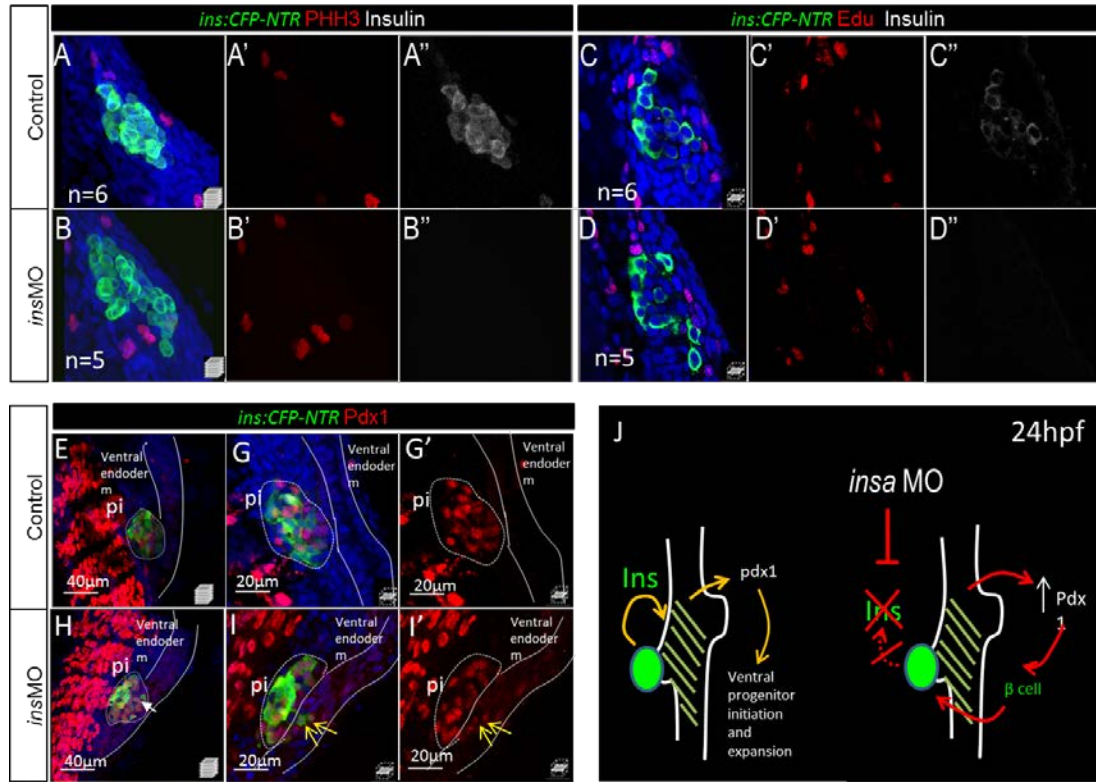


Figure 4-12. *insaMO* does not increase  $\beta$  cell proliferation. (A-B) Confocal projection of 1 dpf control and *insaMO*-injected islets in *Tg(ins:CFP-NTR)* stained with CFP (green), PHH3 (red) and insulin (white). (C-D) Confocal planes of 1 dpf control and *insaMO*-injected islets in *Tg(ins:CFP-NTR)* stained with CFP (green), Edu (red) and Insulin (white). (E-I) Confocal projections and planes of control and *insaMO*-injected endoderm in *Tg(ins:CFP-NTR)* stained for CFP (green) and Pdx1 (red). Note the formation of  $\beta$  cells from  $Pdx1^{+}$  ventral endoderm in *insaMO*-injected endoderm (yellow arrow). pi: principal islet. (J) Model showing how knockdown of insulin might drive  $\beta$  cell formation from the ventral endoderm associated with increased Pdx1 expression.

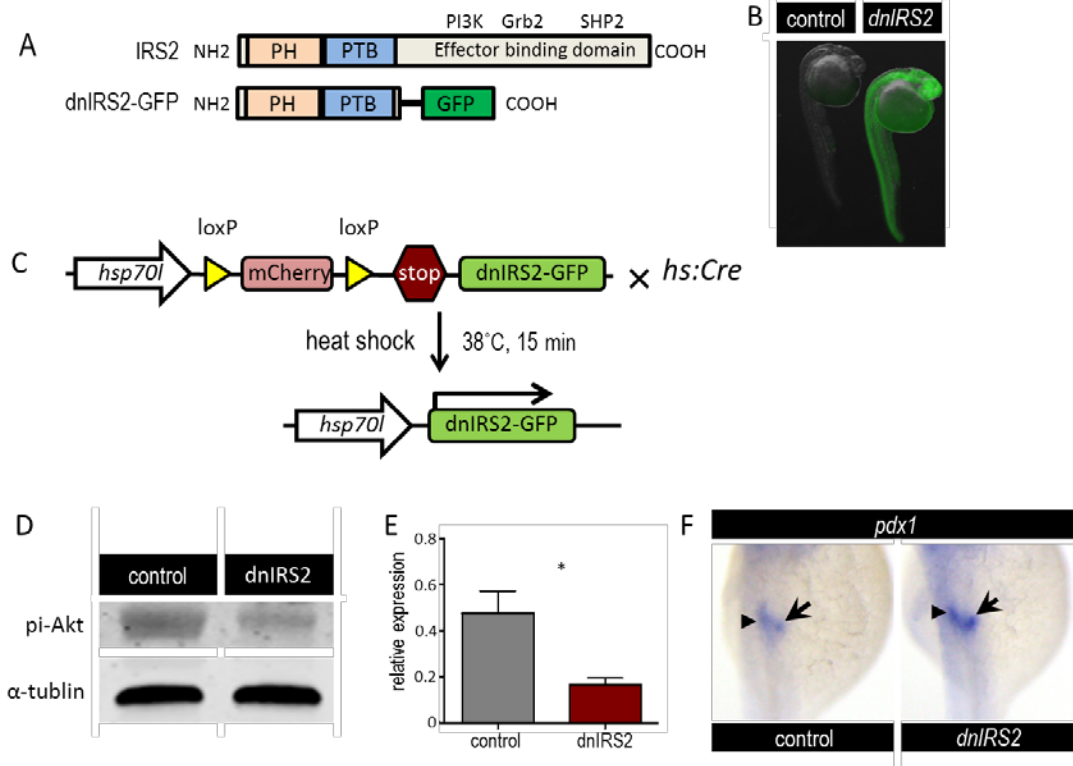


Figure 4-13. Insulin signaling blockade via dnIRS2 mis-expression. (A) Schematic of dominant negative IRS2 (dnIRS2). (B) Control and *dnIRS2-GFP* mRNA-injected embryos at 24 hpf. (C) Scheme of dnIRS2-GFP mis-expression via the HOT-Cre system. *Tg(hs:loxP-mcherry-loxP-dnIRS2GFP)* was crossed with *Tg(hs:Cre)* and *dnIRS2-GFP* mRNA expression was induced upon heat shock induction. (D) Western blot indicating the expression of phospho-Akt 54 hpf in control and dnIRS2 mis-expressing embryos. Control: *Tg(hs:loxP-mcherry-loxP-dnIRS2GFP)<sup>+</sup>Tg(hs:cre)<sup>-</sup>*; *dnIRS2*: *Tg(hs:loxP-mcherry-loxP-dnIRS2GFP)<sup>+</sup> Tg(hs:cre)<sup>+</sup>*. *dnIRS2* were heat-induced at 10 hpf, 24 hpf, 28 hpf, 32 hpf, 36 hpf, and 48 hpf. (E) Quantification of phospho-Akt levels in control (n=6) and dnIRS2 embryos (n=5). Student's t-test was used for statistical analysis. (F) Expression of *pdx1* in 54 hpf control and *dnIRS2* embryos using *in situ* hybridization.



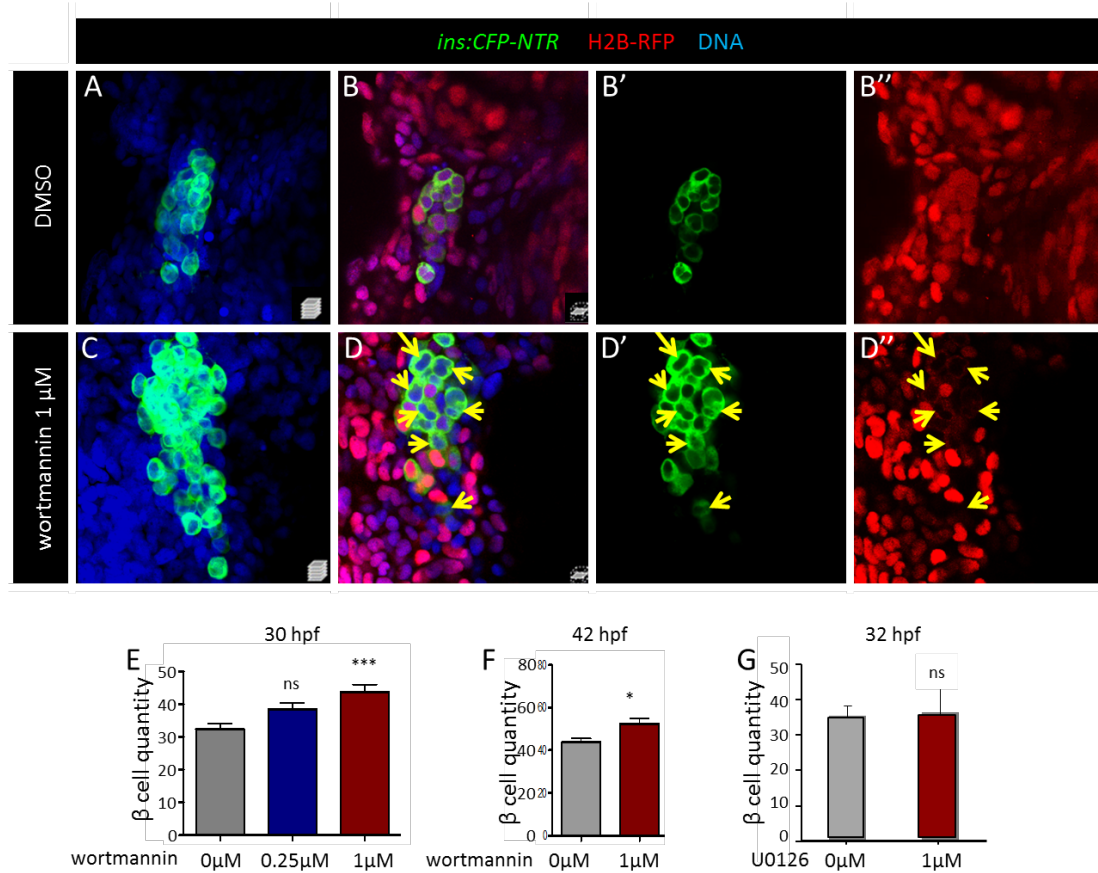


Figure 4-14. Treatment of PI3K inhibitor wortmannin increases  $\beta$  cell differentiation from ventral endoderm. (A-B) Confocal projection of 30 hpf control (A) and 1  $\mu$ M wortmannin-treated (B) islets from *Tg(ins:CFP-NTR)* stained with CFP (green). (C-D) Confocal planes of 30 hpf control (C) and 1  $\mu$ M wortmannin-treated (D) islets with zygotic injection of H2B-RFP mRNA stained with CFP (green). It is noted that majority of control  $\beta$  cells were H2B-RFP<sup>+</sup> but significant portion of H2B-RFP<sup>-</sup>  $\beta$  cells (yellow arrows) can be detected in wortmannin-treated embryos. (E) Quantification of  $\beta$  cell number in DMSO control (n=18), 0.25  $\mu$ M (n=13) and 1  $\mu$ M wortmannin-treated embryos (n=17) at 30 hpf. (F) Quantification of  $\beta$  cells in DMSO control (n=17) and 1  $\mu$ M wortmannin-treated (n=9) embryos at 42 hpf. (G) Quantification of  $\beta$  cell number in DMSO control (n=10) and U0126 treated (n=9) embryos at 32 hpf. U0126 was treated with the dose of 100  $\mu$ M. One-way ANOVA was used in E and Student's t-test was used in F, G for statistical analysis.

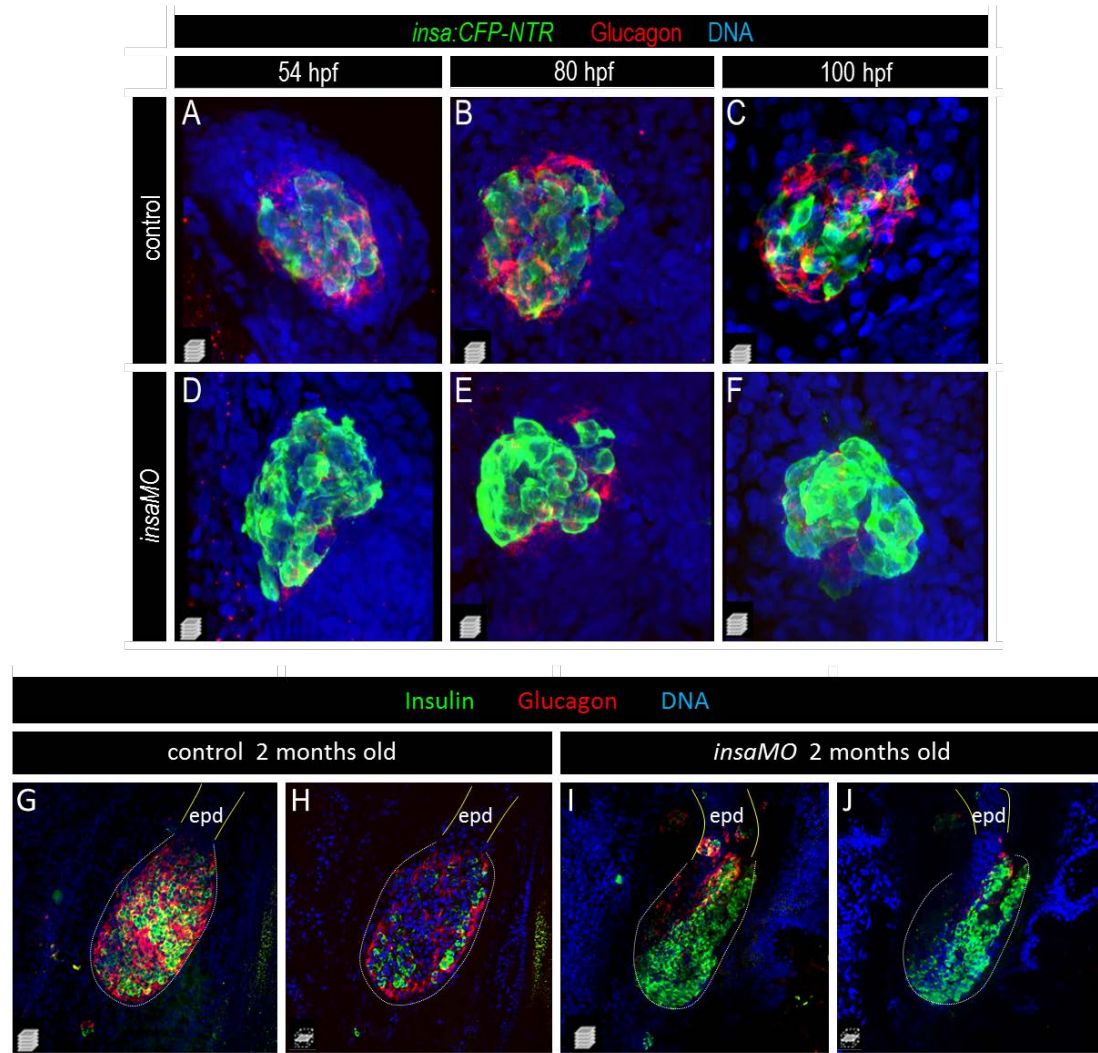


Figure 4-15. Knockdown of insulin influences islet structure and  $\alpha$  cell development. (A-F) Confocal projections of control and *insaMO*-injected islets from *Tg(ins:CFP-NTR)* embryos at 54 hpf, 80 hpf and 100 hpf stained with CFP (green) and glucagon (red). (G,I) Confocal projections of two month old control and *insaMO*-injected islets stained with insulin (green) and glucagon (red). (H,J) Confocal planes of two month old control and *insaMO* injected islets stained with insulin (green) and glucagon (red). It is notable that the islet is consistently dysmorphic in *insaMO*-injected embryos and glucagon<sup>+</sup> cells in *insaMO* primary located near extra-pancreatic duct region.

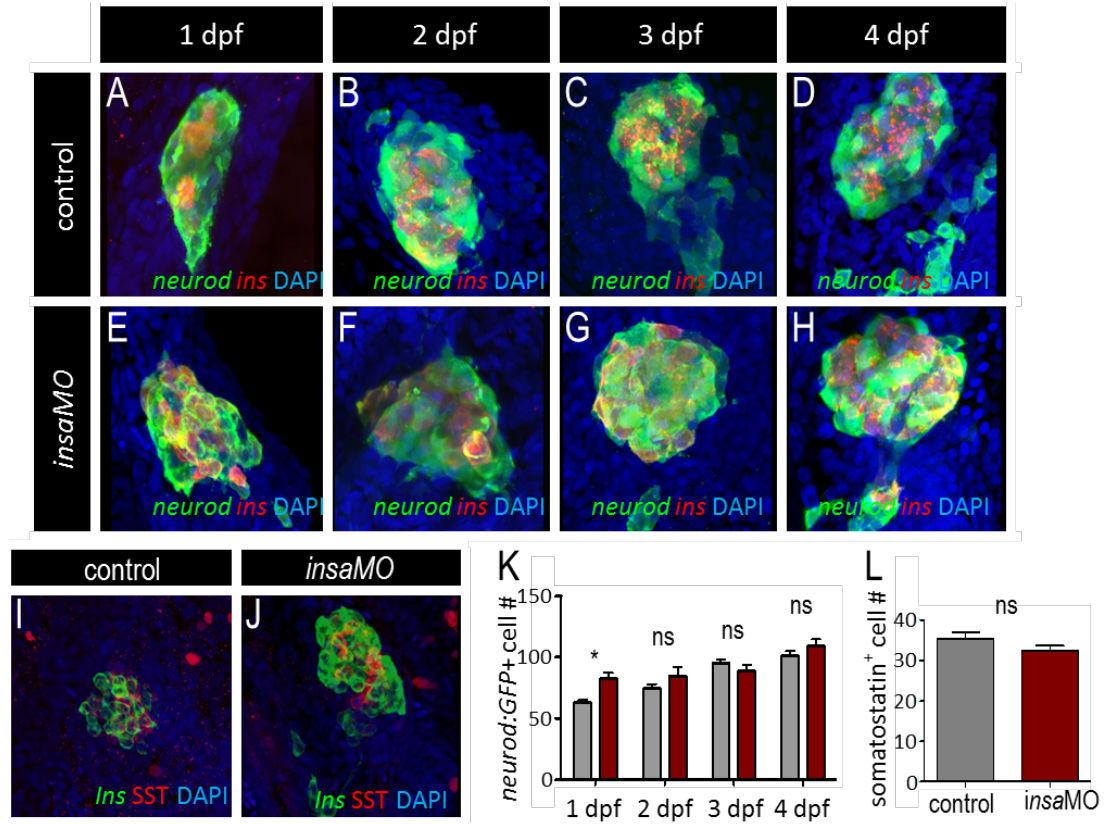


Figure 4-16. The effects of *insaMO* on total endocrine cell number and  $\delta$  cell number. (A-H) Confocal projections of control islets and *insaMO* islets in *Tg(neurod:GFP)Tg(ins:dsRed)* at 1 dpf to 4 dpf. (I-J) Confocal projections of control and *insaMO* injected islets at 3 dpf stained with insulin (green) and somatostatin (red). (K) Quantification of total endocrine cell number in both control and *insaMO* injected embryos from 1 dpf to 4 dpf.  $n \geq 7$  for all the time points. (L) Quantification of total somatostatin<sup>+</sup> cells in both control ( $n=5$ ) and *insaMO* injected ( $n=6$ ) embryos at 3 dpf. Two way ANOVA was used in K and Student's t-test was used in L for statistical analysis.

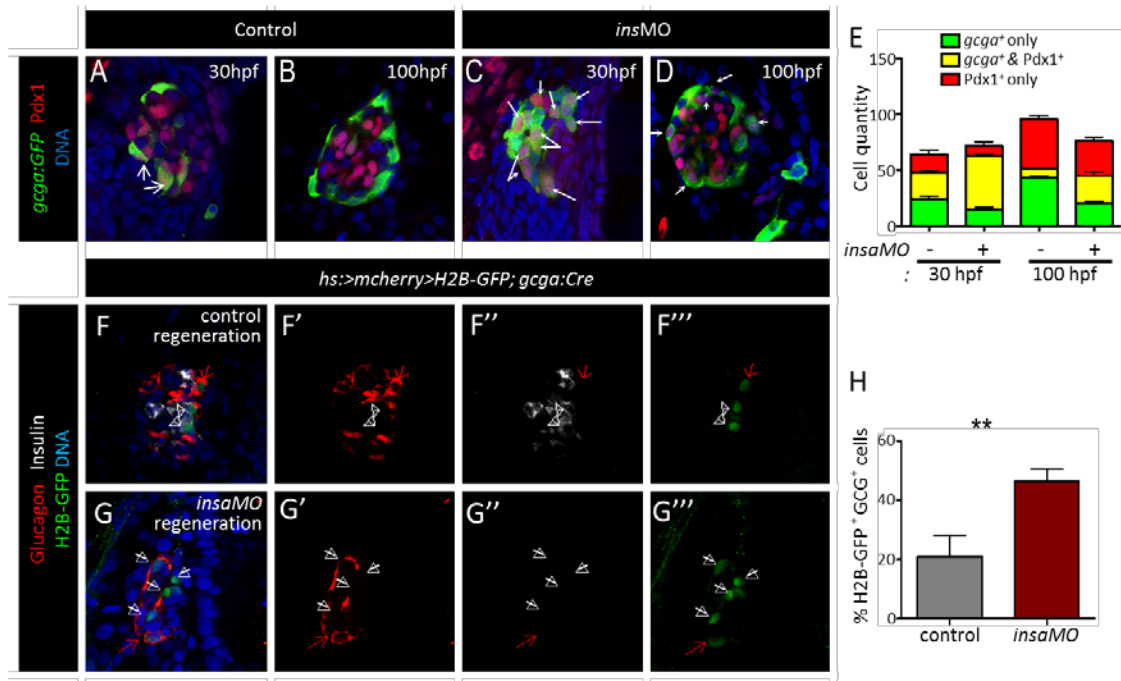


Figure 4-17. Knockdown of *insulin* increases  $\alpha$  cell plasticity. (A-D) Confocal plane image showing both control and *insaMO* injected islets in *Tg(gcga:GFP)* at 30 hpf and 100 hpf stained with Pdx1 (red). (E) Quantification of *gcga:GFP*<sup>+</sup> (green bar), *gcga:GFP*<sup>+</sup> Pdx1<sup>+</sup> (yellow bar), *gcga:GFP*<sup>-</sup> Pdx1<sup>+</sup> (red bar) cell number in both control and *insaMO* injected embryos at 30 hpf and 100 hpf.  $n \geq 7$  for all the time points. (F-G) Confocal plane image showing  $\beta$  cell regeneration islets in control and *insaMO* injected *Tg(ins:Flag-NTR) Tg(hs:loxp-mcherry-loxp-H2BGFP) Tg(gcga:cre)*<sup>s962</sup> embryos stained with Insulin (white), GFP (green) and Glucagon (red). Red arrow showing the H2B-GFP<sup>+</sup> Glucagon<sup>+</sup> cells which represent the  $\alpha$  cell population which retains  $\alpha$  cell fate during  $\beta$  cell regeneration. White arrow showing the H2B-GFP<sup>+</sup> glucagon<sup>-</sup> cells which represent the  $\alpha$  cell population which lost  $\alpha$  cell fate during  $\beta$  cell regeneration. (H) Percentage of H2B-GFP<sup>+</sup> Gcg<sup>-</sup> cell population in both control (n=7) and *insaMO* regenerating (n=13) islet. Student's t-test was used for the statistical analysis in H.



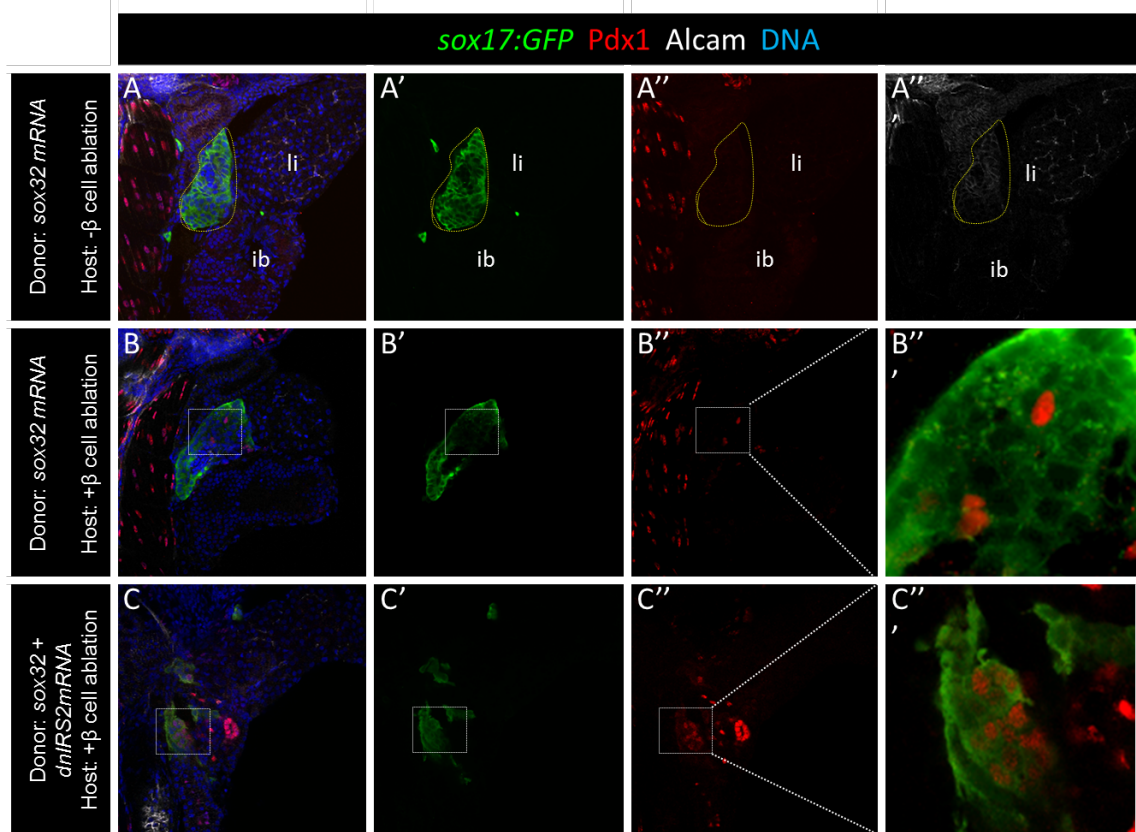


Figure 4-18. Inhibition of insulin signaling increases Pdx1 induction in transplanted donor blastula stem cells (BSCs). (A) Confocal plane of control donor *Tg(sox17:GFP)* BSCs transplanted into host larvae without  $\beta$  cell ablation at 1 day post transplantation stained with Pdx1(red) and Alcam (white). (B) Confocal plane of control donor *Tg(sox17:GFP)* BSCs transplanted into host larvae with  $\beta$  cell ablation at 1 day post transplantation stained with Pdx1(red). (C) Confocal plane of donor *Tg(sox17:GFP)* BSCs injected with dnIRS2-GFP mRNA and then transplanted into host larvae with  $\beta$  cell ablation at 1 day post transplantation stained with Pdx1(red).

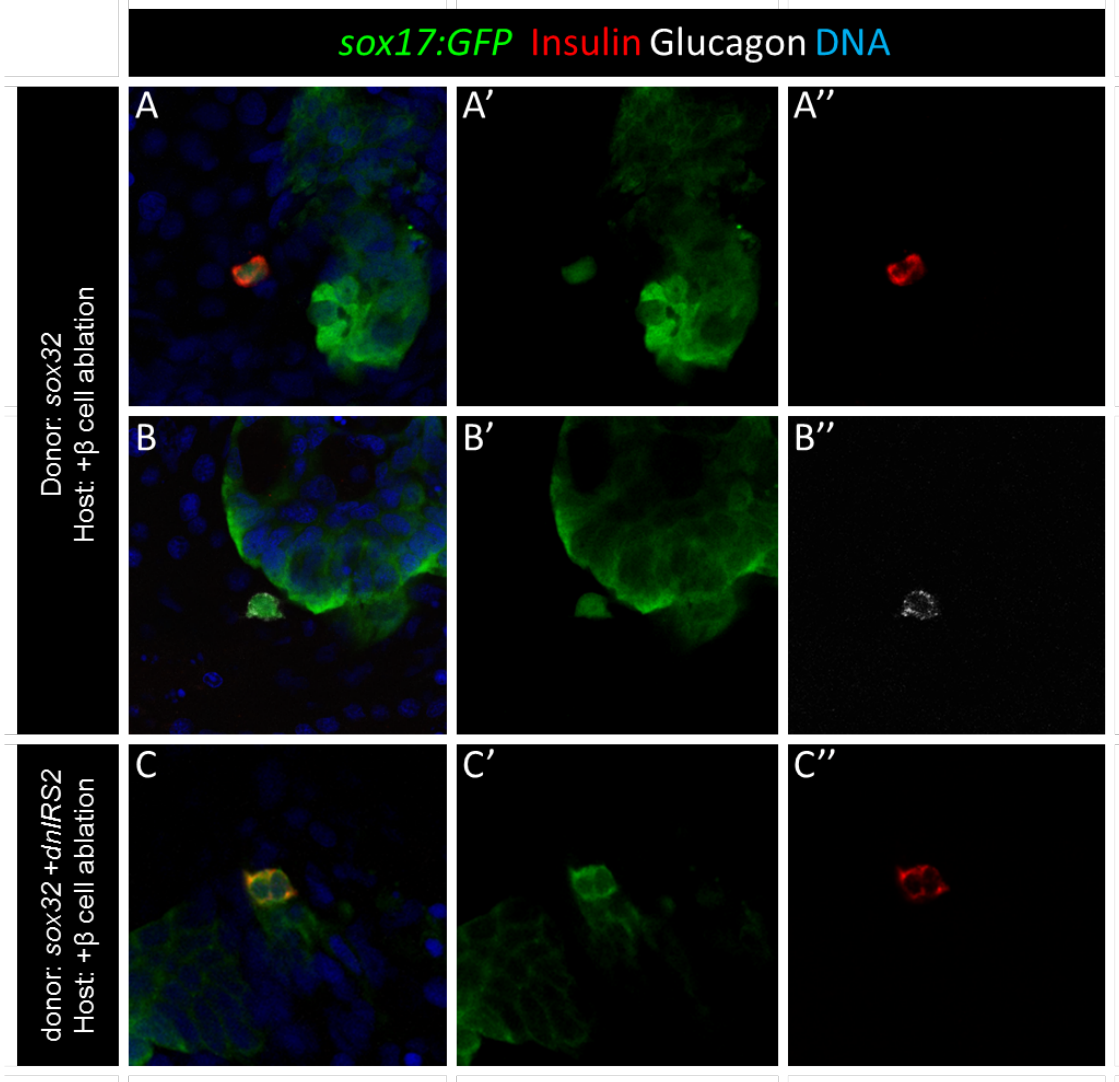


Figure 4-19. Transplanted donor BSCs can be induced to differentiate into pancreatic endocrine cells in  $\beta$  cell ablated host larvae. (A-B) Confocal plane images of Confocal donor *Tg(sox17:GFP)* BSCs transplanted into host larvae with  $\beta$  cell ablation at 2 day post transplantation stained with Insulin(red) and Glucagon(white). (C) Confocal plane of donor *Tg(sox17:GFP)* BSCs injected with *dnIRS2-GFP* mRNA and then transplanted into host larvae with  $\beta$  cell ablation at 2 day post transplantation stained with Insulin(red) and Glucagon(white).

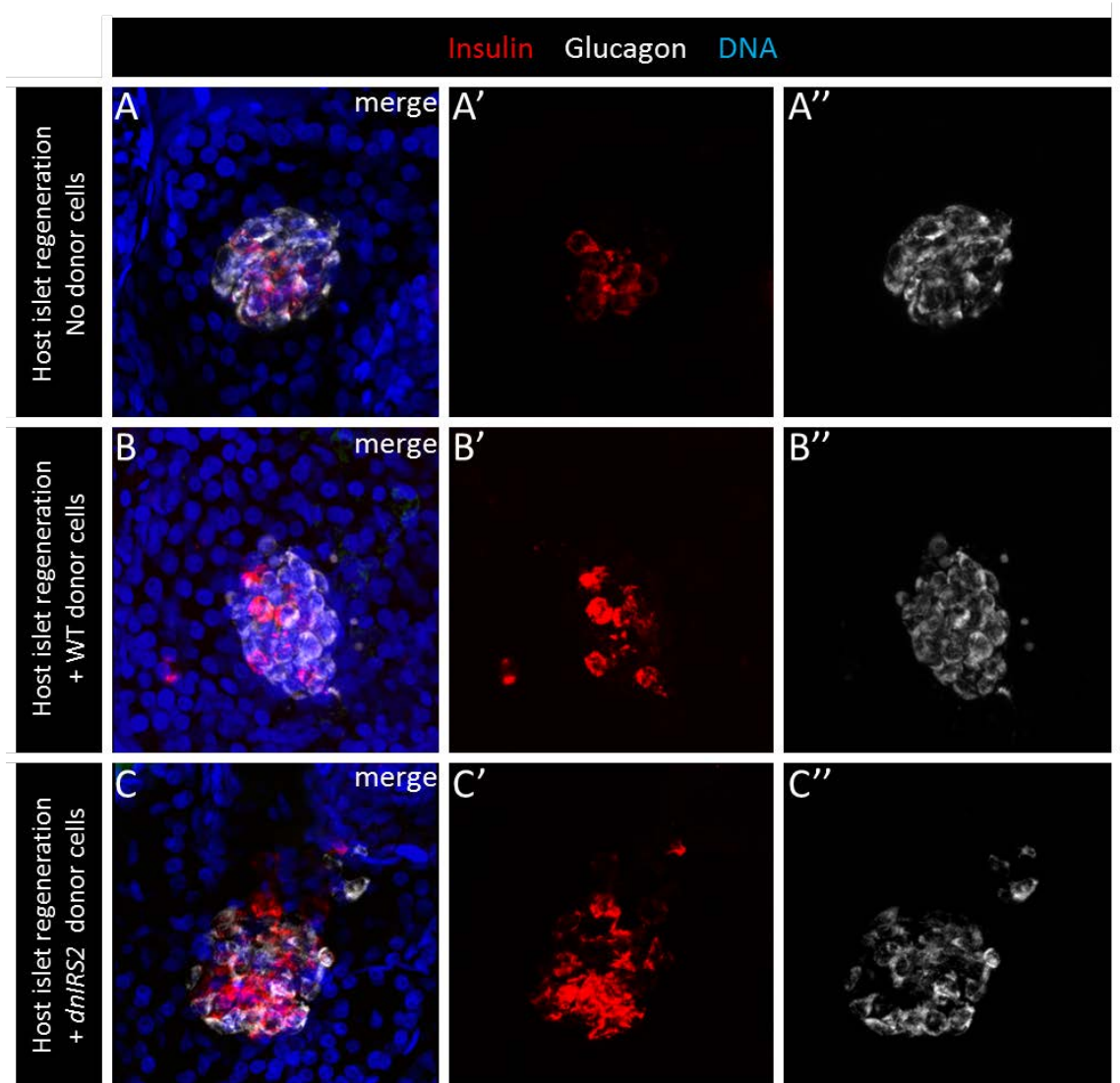


Figure 4-20. Transplantation of BSCs increases host  $\beta$  cell regeneration. (A-C) Confocal projection of 2 day post ablation regenerating host islets without transplantation (sham control, A), transplanted with control *Tg(sox17:GFP)* donor BSCs (B) and transplanted with *Tg(sox17:GFP)* *dnIRS2*-GFP mRNA injected donor BSCs(C) stained with Insulin(red) and Glucagon(white).

## **CHAPTER FIVE: Insulin signaling is required for exocrine pancreas formation and acinar cell fate maintenance**

### **V.A. Summary**

As an integrated organ, the function of the pancreatic exocrine cells is highly regulated by pancreatic endocrine hormones including insulin, which is secreted from islet  $\beta$  cells. However, the impact of the pancreatic hormones on the exocrine pancreas during embryonic development is unclear. It is known that both pancreatic endocrine and exocrine components are derived from the same progenitor cells during development. Results in Chapter Four have revealed that insufficient insulin signaling during early development induce the precocious differentiation of pancreatic progenitors into  $\beta$  cells. However, the effect of insulin signaling on exocrine pancreas development is still unknown. In this study, using multiple genetic approaches, we have revealed that insulin signaling is critical for exocrine pancreas development. Insufficient insulin signaling during early embryonic development will impair pancreatic progenitor cell differentiation toward the exocrine cell lineage. Moreover, the impairment in exocrine pancreas persists until adulthood, indicating that the early loss of insulin signaling permanently altered the pancreatic differentiation program. Using a unique Hot-cre inducible transgenic system, we have shown that specific blockage of insulin signaling in differentiated acinar cells will induce the expression of the pancreatic progenitor and duct cell marker Pdx1. These data suggest that insulin signaling may be critical for maintaining the mature acinar cell fate. Altogether, our studies show that insulin signaling is not only important in regulating exocrine pancreas functions but is also indispensable to ensure the appropriate development of the exocrine pancreas.

### **V.B. Introduction**

The pancreas is a compound organ that facilitates macronutrient breakdown and absorption and regulates whole body metabolism. In order to fulfill these complex roles, the pancreas is composed of both exocrine and endocrine components (44). The majority



of pancreatic tissue belongs to the exocrine component, which is made of the duct epithelium cells and acinar cells. Digestive enzymes and pancreatic juices are synthesized in acinar cells and then secreted into the pancreatic duct system, which is connected to the duodenum. Upon entering the intestine, these enzymes become activated to aid digestion. On the other hand, the pancreatic endocrine hormones are secreted from the islets of Langerhans, which are dispersed throughout the pancreatic exocrine tissue. Insulin is one of the key islet hormones; it is secreted from islet  $\beta$  cells and plays a fundamental role in regulating glucose homeostasis.

Previous studies have shown that the cellular functions of the exocrine pancreas are directly regulated by insulin released from the islet, indicating the existence of an islet-exocrine axis (137). As acinar cells receive blood flow directly from the islet via the portal vein system, these cells are exposed to some of the highest insulin levels among all somatic cells (365), and consistent with this observation, insulin regulates the expression of insulin receptor on the surface of acinar cells (366). Moreover, amylase mRNA levels progressively decrease in an insulin-deficient, STZ-induced, rat model of  $\beta$  cell ablation; this effect can be reversed by insulin treatment (367). Results from both *in vitro* and *in vivo* studies have revealed that insulin potentiates cholecystokinin-induced pancreatic exocrine secretion (368,369). Important roles of insulin signaling in exocrine pancreas growth and function have also been revealed by clinical studies. Insufficient insulin signaling causes an increase in blood glucose, and can subsequently result in the development of diabetes mellitus. Type 1 Diabetes Mellitus (T1DM) is caused by autoimmune destruction of  $\beta$  cells, which results in absolute insulin deficiency. Clinical studies have demonstrated that acini surrounding insulin-containing islets are larger and contain more zymogen granules than acini surrounding insulin deficient islets, indicating that insulin can act as a local factor to directly regulate acinar enzyme synthesis and secretion in humans (370). Moreover, a decrease in exocrine pancreas size is correlated with T1DM (371-373). Furthermore, it is reported that the size of pancreas in children (<15 years old) with T1DM is correlated with duration of disease progress and severity of insulin deficiency (374-376). Together these clinical studies demonstrate that insulin may not only play important roles in the regulation of exocrine pancreas function but also in exocrine pancreas development and growth. However, our understanding of the influence

of insulin signaling on exocrine pancreas development in the embryonic period remains meager.

Endocrine cells, including the insulin-secreting  $\beta$  cells, and exocrine cells are derived from common progenitors that co-express two key transcription factors: Pdx1 and Ptf1a (48). The differentiation potential of these progenitors is highly regulated both by their environment and their innate genetic programming (181). In this study, we have used the zebrafish as a model (216,221) to study the influence of insulin signaling on exocrine pancreas development. Previous studies have shown that zebrafish and mammals have highly conserved metabolic profiles and pancreatic developmental programming (216,221). In addition, pancreas development is largely similar. As in mammals, the zebrafish pancreas is derived from two progenitor cell domains, the dorsal and ventral pancreatic buds (220). The zebrafish dorsal bud differentiates early, and gives rise solely to endocrine cells. These cluster to form the principal islet by 24 hours post fertilization (hpf). The zebrafish ventral bud, on the other hand, gives rise to both exocrine and endocrine cells. As in mice and humans, the zebrafish ventral pancreas progenitors initiate as multipotent progenitors that are marked by Pdx1 and Ptf1a expression (183,227,228). After their induction from the ventral endoderm, these progenitor cells migrate, and engulf the dorsal bud-derived cells at 34 hpf. As exocrine pancreas development progresses, *ptf1a* is excluded from duct cells, but remains expressed in acinar cells where it is crucial for acinar cell functions (72). In mature acinar cells, Ptf1a complexes with other transcription factors to drive the expression of acinar cell functional genes, such as *elastase* and *trypsin* (377). On the other hand, the expression of *pdx1* is selectively activated in duct cells, but not acinar cells (223).

In zebrafish larvae, the primary energy source transitions from yolk to extragenous nutrients; this is associated with the functional maturation of exocrine pancreas and is marked by digestive enzyme secretion from acinar cells (1,183). The coincident timing of these transitions suggests that metabolic signals are involved in the regulation of exocrine pancreas development and maturation. As we have shown in Chapter Four, insulin signaling plays an important role in regulating pancreatic progenitor cell differentiation. Loss of insulin signaling resulted in the precocious differentiation of pancreatic progenitor cells into  $\beta$  cells. Since both pancreatic exocrine and endocrine

components are derived from the same progenitor pool, we therefore hypothesized that disruptions to the early progenitor cell differentiation program resulting from insulin deficiency would impair the development and maturation of exocrine pancreas.

In this chapter, we have analyzed the insulin-exocrine pancreas axis during pancreas development. Using multiple genetic approaches and the zebrafish as a model, we show that insulin signaling during early pancreas development drives pancreatic progenitor differentiation toward the exocrine cells fate. Moreover, our data reveal a critical period for insulin function during pancreatogenesis, as defects arising from insulin signaling impairment persist in adult animals. Finally, insulin signaling suppresses Pdx1 expression in differentiated acinar cells, and thereby contributes to the maintenance of acinar cell fate. Further elucidation of the mechanisms by which insulin regulates exocrine pancreas development will contribute to the understanding of T1DM-associated exocrine pancreas insufficiency.

## **V.C. Results**

### **Insufficient insulin signaling impairs exocrine pancreas development.**

In order to investigate how insulin signaling impacts pancreatic exocrine cell development, we used an *inulin* morpholino (*insaMO*) to knockdown *insulin a* mRNA. As shown in Chapter Four, *insaMO* is specific and efficacious, and knockdown of insulin via *insaMO* blocks insulin synthesis from pancreatic  $\beta$  cells. To visualize exocrine cell development, we used *Tg(ptf1a:GFP)*, which is shown to be expressed in the retina, brain and pancreatic domain (183). Using this approach, we found that injection of *insaMO* strongly impaired exocrine pancreas development at 4 dpf (Figure 5-1A). However, there was no significant difference of *ptf1a:GFP* expression in the hindbrain and retina, indicating this may be a pancreas-specific role for *insa* (Figure 5-1A). In both mammals and zebrafish, ventral pancreas progenitors are derived from bi-potent endoderm progenitors that also gives rise to liver (220). Thus, we also investigated if knockdown of insulin influenced liver development using the transgenic fish line *Tg(fabp10:dsRed);Tg(ptf1a:GFP)*, in which hepatocytes were marked by red fluorescence and acinar cells were marked with green fluorescence (Figure 5-6). We

observed that although *insa* knockdown strongly impaired pancreas development, the liver of *insaMO* embryos appeared grossly similar to controls (Figure 5-6A, B). This indicated that insulin signaling likely does not regulate the development of all endoderm, but rather acts as a local factor to regulate pancreas development. To further probe this pancreatic role, the acinar cell-specific marker *trypsin* was analyzed by *in situ* hybridization (ISH) and real time PCR. We found a significant decrease in *trypsin* expression in the *insaMO*-injected embryos at 3 and 4 dpf as compared to controls (Figure 5-1B). Moreover, real time PCR data revealed a 40% decrease in *trypsin* expression in the *insaMO*-injected embryos (Figure 5-1C).

Next, we used the HOT-Cre system of inducible transgenes to block insulin signaling by overexpression of dominant-negative *insulin receptor substrate 2* (*dnIRS2*). In this synthetic construct, the C-terminal effector binding domain of *IRS2* was substituted with GFP, which resulted in the preservation of binding affinity to the insulin receptor, but blocked transmission of insulin signaling further downstream (Figure 5-1D). Using this system, we crossed *Tg(hs:cre)* (hereafter *hs:cre*) with *Tg(hs:loxp-mcherry-loxp-dnIRS2GFP)* (hereafter *dnIRS2*), to generate embryos that could overexpress *dnIRS2* and block insulin signaling upon heat shock induction (Figure 5-1E). The efficiency of insulin signaling blockade by *dnIRS2* was verified as described in Chapter Four. We used Alcam immunostaining to visualize the exocrine pancreas, and observed that it was significantly smaller in *dnIRS2* embryos than in controls (Figure 5-1F-G). In order to confirm the defect of exocrine pancreas development, we analyzed *trypsin* expression via ISH at 72 hpf, and found that it was strongly reduced in *dnIRS2*-expressing embryos (Figure 5-6C-D).

Next, we tested whether ablation of  $\beta$  cells influenced exocrine pancreas development. For this, we used the *Tg(gata5:loxp-mcherry-loxp-DTA)* transgenic line together with *Tg(insa:cre)* to generate embryos in which 48% of  $\beta$  cells were specifically destroyed by Diphtheria Toxin A (DTA; Figure 5-6C-E). By 72 hpf,  $\beta$  cell-ablated embryos showed a 20% decrease in exocrine pancreatic length as compared to control marked by Alcam (Figure 5-6F).

### ***Insulin* knockdown impairs exocrine pancreas differentiation rather than proliferation.**

Since Insulin has been proposed as an important mitogen that stimulates proliferation (378), we then reasoned that the decrease in exocrine pancreas size in response to insulin deficiency might be due to a decreased exocrine pancreas proliferation. To test this, we first analyzed the proliferation rate of the exocrine pancreas via immuno-staining for the M-phase cell cycle marker phosphohistone H3 (PHH3). We observed that although there was a significant decrease in exocrine pancreas mass in *insaMO*-injected endoderm, we found that after normalizing to total *ptf1a:GFP*<sup>+</sup> cell number, the PHH3<sup>+</sup> *ptf1a: GFP*<sup>+</sup> cell percentage in *insaMO* injected embryos was not changed at 48 hpf or 74 hpf, suggesting that the proliferation rate of exocrine cells was not decreased in response to the insulin knockdown (Figure 5-2A-C). To further confirm this result, we analyzed cell proliferation by EdU incorporation, which labels replicating DNA. Similarly, we did not observe a difference in the EdU<sup>+</sup> *ptf1a: GFP*<sup>+</sup> cell ratio between control and *insaMO* groups (Figure 5-2D-F). By the concordant data resulting from both approaches, we can exclude the possibility that the impairment of exocrine pancreas growth is due to altered pancreas proliferation. Since insulin is also an anti-apoptotic factor in pancreas (379), we also tested whether *insulin* knockdown increased the rate of apoptosis in the exocrine pancreas. For this, we used cleaved-Caspase 3 immunostaining. Metronidazole-induced  $\beta$  cell apoptosis in *Tg(ins:CFP-NTR)* embryos was used as a positive control (254)(Figure 5-7A). We observed no cleaved-Caspase3<sup>+</sup> acinar cells in either control or *insaMO*-injected pancreata, indicating that inhibition of insulin signaling did not increase acinar cell death (Figure 5-7B-C).

Since the knockdown of *insulin* affected neither proliferation nor apoptosis rates in the exocrine pancreas, we suspected that exocrine pancreas deficiency in *insaMO*-injected embryos might be due to impaired differentiation. To analyze this, *ptf1a: GFP*<sup>+</sup> cells in the ventral pancreas were traced over time. In accord with previous work (183), we found that, *ptf1a: GFP*<sup>+</sup> cells were first detected around 33 hpf in the ventral endoderm of control embryos, and that these newly derived *ptf1a: GFP*<sup>+</sup> cells were close to the dorsal pancreatic islet (Figure 5-3A). We found that after their induction from the ventral endoderm, these *ptf1a: GFP*<sup>+</sup> cells expanded until they merged with the dorsal

endocrine cells (Figure 5-3A-F). Inhibition of insulin signaling via *insaMO* delayed the induction of *ptf1a: GFP*<sup>+</sup> cells. The first *ptf1a: GFP*<sup>+</sup> cells in *insaMO*-injected embryos were detected at 38 hpf near the anterior region of the dorsal bud, and these *ptf1a: GFP*<sup>+</sup> cells failed to merge with the principal islet at 61 hpf (Figure 5-3G-M, P). The delayed appearance of *ptf1a: GFP*<sup>+</sup> cells was not likely due to a general delay of development, as *insaMO*-injected embryos exhibited no general developmental defects (see also chapter four). Furthermore, our results indicate that *insa* knockdown not only delayed the initiation of *ptf1a: GFP*<sup>+</sup> cells but also decreased the overall *ptf1a: GFP*<sup>+</sup> cell quantity (Figure 5-3G-M, P).

Both Ptf1a and Pdx1 are expressed in early pancreatic progenitor cells (227,228). During murine exocrine pancreas development, Pdx1 is repressed in the acinar cell lineage, but is continuously expressed in duct cells (380,381). In accord with previous findings, we found that Pdx1 expression in adult zebrafish exocrine pancreas was also limited to the duct cells (Figure 5-8). On the other hand Ptf1a is expressed only in acinar cells, and not duct cells (382). In order to investigate the dynamic expression of Pdx1 and Ptf1a during pancreatic progenitor differentiation, we performed immunostaining for Pdx1 in *Tg(ptf1a:GFP)* embryos. We found that in 33 hpf control pancreata, all *ptf1a: GFP*<sup>+</sup> cells were also Pdx1<sup>+</sup> (Figure 5-3A-B, Q). However, in 44 hpf control embryos, there were three cell classifications in ventral bud derived pancreas: (1) Pdx1<sup>+</sup> *ptf1a:GFP*<sup>+</sup>, (2) Pdx1<sup>+</sup> *ptf1a:GFP*<sup>-</sup> and (3) Pdx1<sup>-</sup> *ptf1a:GFP*<sup>+</sup> (Figure 5-3C-D, Q). In contrast to control embryos, all *ptf1a:GFP*<sup>+</sup> cells were also found to be Pdx1<sup>+</sup> in 44 hpf *insaMO*-injected embryos (Figure 5-3I-J, Q). Moreover, in 61 hpf control embryos, only *ptf1a:GFP*<sup>+</sup> Pdx1<sup>-</sup> and Pdx1<sup>+</sup> *ptf1a:GFP*<sup>-</sup> cells, but not *ptf1a:GFP*<sup>+</sup> Pdx1<sup>+</sup> cells, were detectable (Figure 5-3E-F, Q). Yet, in 61 hpf *insaMO*-injected embryos, 67% of *ptf1a:GFP*<sup>+</sup> cells were found to co-express Pdx1.

Normally, during exocrine pancreas development, Pdx1 is selectively activated in the duct cell lineage (380). We therefore hypothesized that in the exocrine pancreas of 61 hpf *insaMO*-injected embryos that the Pdx1<sup>+</sup> *ptf1a:GFP*<sup>+</sup> cells represented mis-differentiated acinar cells that also exhibited duct cell characteristics. To test this, we performed immuno-staining with 2F11 in *Tg(ptf1a:GFP)* embryos. 2F11 antibody marks zebrafish pancreatic duct cells, and the 2F11<sup>+</sup> domain overlaps the pancreas duct marker *sox9b*

(383). Furthermore, 2F11 is expressed in a complementary pattern to *ptfla: GFP*<sup>+</sup> cells (Figure 5-3N). This is consistent with our observations that *ptfla: GFP*<sup>+</sup> cells do not express Pdx1 at 61 hpf (Figure 5-3E, F, Q). However, in 72 hpf *insaMO*-injected pancreas, we found that majority of *ptfla: GFP*<sup>+</sup> cells co-expressed 2F11. Furthermore, Notch signaling is repressed during acinar cell differentiation, but is maintained in differentiated pancreatic duct cells (111). We thus used the Notch reporter transgenic line *Tg(tp1:nls-Red)* to analyze whether *insa* knockdown could induce formation of acinar cells that inappropriately activate Notch signaling. Consistent with the findings above, we detected *tp1:nls-Red*<sup>+</sup> *ptfla:GFP*<sup>+</sup> cells in 72 hpf *insaMO*-injected pancreas, but not in control pancreata (Figure 5-9).

Altogether, we have shown that in the absence of insulin, zebrafish ventral pancreatic progenitor cells cannot differentiate appropriately into acinar cells and duct cells. Furthermore, *insulin* knockdown results in confused exocrine cell types, which co-express both acinar and duct cell genes (Figure 5-3R).

### **Inhibition of Insulin signaling activates Pdx1 expression in differentiated acinar cells**

Thus far, we have shown that inhibition of insulin signaling impairs the exocrine pancreas developmental program. We next asked whether insulin signaling directly influenced acinar cells during development. To test this, we analyzed the expression of the insulin receptor in exocrine pancreas using ISH. There are two isoforms of the insulin receptor in zebrafish, *insra* and *insrb*. Our results indicated that both genes are expressed in the exocrine compartment, and are especially highly expressed in acinar, but not duct cells at 72 hpf (Figure 5-10). The presence of insulin receptors in acinar cells indicates that insulin signals secreted from pancreatic  $\beta$  cells may directly act on acinar cells during development.

In order to understand how insulin signaling influences differentiated acinar cells, we blocked insulin signaling in all differentiated *elastase*-expressing acinar cells. For this, we crossed *dnIRS2* fish with *Tg(ela3l:cre; cryaa:YFP)* (hereafter *ela:cre*) fish to induce *dnIRS2* in acinar cells upon heat shock induction. *Tg(hs:loxp-mcherry-loxp-stop-H2B-*

*GFP*) (hereafter *hs:CSH*);*ela:cre* embryos were used as controls.. Our results showed that there was no significant change in pancreatic size between control and *dnIRS2*; *ela:cre* embryos (Figure 5-5C,E). This is consistent with our finding above that knockdown of *insulin* does not influence acinar cell proliferation or death. However, we observed that blockade of insulin signaling resulted in altered acinar cell morphology. Using Alcarn to mark the border of exocrine pancreas cells in 84 hpf control embryos, we found that acinar cells were much larger than pancreatic duct cells and acinar cellular nuclei were located basally (Figure 5-4A). However, after insulin signaling blockade with *dnIRS2*; *ela:cre*, we could no longer distinguish acinar cells by morphology (Figure 5-4E,F).

Next we analyzed Pdx1 expression and found that in the exocrine pancreas at 84 hpf, Pdx1 expression was detected in 2F11+ duct cells, but not acinar cells (Figure 5-5A-B). However, after insulin signaling blockade using *dnIRS2GFP*; *ela:cre*, we found that many *dnIRS2-GFP*<sup>+</sup> acinar cells expressed Pdx1 after heat shock induction (Figure 5-5E-F). In contrast, in the control *ela:cre*; *hs:CSH* embryos, Pdx1 was completely excluded from H2B-GFP labeled acinar cells (Figure 5-4C). Pdx1 has a key role in promoting pancreatic  $\beta$  cell formation and the misexpression of *Pdx1* in pancreatic  $\alpha$  cells can induce  $\alpha$  to  $\beta$  cell transdifferentiation (65,271). With this data in mind, we hypothesized that activation of Pdx1 in acinar cells would promote acinar to  $\beta$  cell transdifferentiation. To test this hypothesis, we examined Insulin expression in 72 hpf *dnIRS2*; *ela:cre* embryos after heat shock induction, but did not detect any *dnIRS2-GFP*<sup>+</sup> cells that co-expressed Insulin protein (Figure 5-11A). Next, to address whether acinar to  $\beta$  cell transdifferentiation could be induced by combining the conditions of insulin signaling deficiency and  $\beta$  cell loss, we ablated  $\beta$  cells in *ela:cre*; *dnIRS2*; *ins:Flag-NTR* transgenic embryos. However, we found that following metronidazole-induced  $\beta$  cell ablation (271), regenerating  $\beta$  cells were only observed in the islet region, and that no ectopic *dnIRS2-GFP*<sup>+</sup> Insulin<sup>+</sup> cells were detected at 1 day post ablation (Figure 5-11B). These results indicate that derepression of Pdx1 in acinar cells it is not sufficient to promote their transformation into  $\beta$  cells. These data indicate that blockade of insulin signaling in acinar cells activates Pdx1 expression, and suggest that insulin normally serves to suppress acinar cell expression of Pdx1.



## **Diminished insulin signaling during pancreatogenesis permanently alters exocrine pancreas morphology**

In order to explore the long term effects of insulin signaling blockade on exocrine pancreas development, we aged control and *insaMO*-injected embryos to 2 months post fertilization (mpf), and then analyzed the morphology of the exocrine pancreas. For this we used fish bearing the transgene *Tg(fabp10:dsRed;ela3l:GFP)*, in which hepatocytes are marked with red and acinar cells with green fluorescence. Morpholinos become diluted as cells divide and this diminishes their capacity for genetic knockdown (261). We therefore questioned whether restoration of *insulin* expression would stimulate exocrine pancreas regeneration. Externally, *insaMO*-injected adult fish were indistinguishable from controls (Figure 5-5A). Internally, in control fish, the head of the pancreas is positioned on the right side (as seen from the dorsal aspect) and the principal islet is located at the anterior of the pancreatic head, which is usually covered by liver (Figure 5-5B, D). The zebrafish pancreas continues to grow throughout adult stages (384), and multiple pancreatic tails extend along the intestinal loops (Figure 5-5B,D, J). A distribution of smaller secondary islets is found along each of these tail lobes (Figure 5-5D). Surprisingly, we found that pancreas morphology was greatly disrupted in *insaMO*-injected fish. In *insaMO*-injected fish, we observed that the exocrine pancreas near the principal islet was severely diminished (Figure 5-5C,G,H,K). In addition, we found that the endocrine morphology was also strongly influenced by *insaMO*. While in control pancreas the secondary islets were scattered throughout the exocrine tissues (Figure 5-5D-F), in 2 mpf *insaMO*-injected pancreata we observed “naked” secondary islets that were not surrounded by acinar cells (Figure 5-5G,I; Figure 5-12A). Furthermore, in control pancreas, both principal islet and secondary islets were comprised of  $\beta$  cells and  $\alpha$  cells (Figure 5-5E-F). However, in *insaMO*-injected pancreata, the  $\alpha$  cell population was severely decreased in the principal islet and was absent from naked secondary islets (Figure 5-5H). This was consistent with our earlier findings (Chapter Four), which showed that Insulin deficiency could promote biased, compensatory  $\beta$  cell differentiation at the expense of  $\alpha$  cell fate.

Interestingly, in *insaMO*-injected fish, we observed discrete segments of pancreas that are not contiguous along the intestine (Figure 5-5C,G,K). We occasionally observed

secondary islets that were surrounded by acinar cells in these segments (Figure 5-12B,C), and the alpha cell content in these islets also appeared to be similar to control (Figure 5-12B,C). We speculate that this is due to pancreas regeneration that occurs after relieving the insulin signal blockade (more discussion below). Nevertheless, the existence of these extra-pancreatic segments may rescue the general development and glucose metabolism of *insaMO*-injected zebrafish.

## V.D. Discussion

In this study, we used different genetic approaches to demonstrate that insulin signaling is critical for exocrine pancreas formation and maturation, as well as maintaining the differentiated acinar cell fate. Our data demonstrate that loss of insulin signaling, using either *insulin* knockdown or *dnIRS2* over-expression, resulted in the impairment of exocrine development. This included a decrease in exocrine pancreas size and in expression of genes encoding digestive enzymes. Moreover, insufficient insulin signaling resulted in decreased progenitor cell differentiation into exocrine lineages, which subsequently resulted in permanent diminution of the exocrine pancreas in adult fish. Finally, our results showed that specifically blocking insulin signaling in acinar cells induced Pdx1 activation and altered acinar cell morphology. These results indicate that insulin signaling not only acts as a development signal to promote acinar cell formation but also plays an important role in sustaining acinar cell fate.

From the studies in Chapter Four, we have revealed an important role for insulin in regulating pancreatic progenitor cell differentiation. Loss of insulin signaling was found to induce precocious differentiation of progenitor cells into  $\beta$  cells, which then depleted the pancreatic progenitor cell pool. Here, in consistent with our previous findings, our results demonstrated that after *insulin* knockdown, the initiation of *ptfla* expression cells in the ventral endoderm requires insulin signaling. In the absence of insulin signaling, the induction of ventral *ptfla* expressing cells was delayed and the quantity of *ptfla* expressing cells was decreased. Therefore, the simplest interpretation of these data is that the impairment of exocrine pancreas development resulting from insulin insufficiency was due to the loss of pancreatic progenitors. This is consistent with previous studies that

showed that loss of Notch signaling, which is crucial for regulation of pancreatic progenitor differentiation, induces premature differentiation of progenitors and depletes the pool of pancreatic precursor cells; this results in a shortage of progenitor cells that can differentiate into exocrine cells (106,107). Here, we have also demonstrated that insufficient insulin signaling during embryogenesis affects the adult pancreas. We observed a severe defect in exocrine pancreas adjacent to the principal islet, and found that the relative quantity of  $\alpha$  cells in the islet near the pancreatic head was strongly reduced. This is consistent with the observation that the adult pancreatic organ size is determined by the mass of early pancreatic progenitors (115). Interestingly, in our model, we observed the formation of discontinuous segments of pancreas which exhibited normal morphology. Although it is unclear in mammals whether pancreatic progenitors persist in adult pancreas (176,179,275), it is clear in zebrafish that pancreatic progenitors persist and retain their differentiation capacity until adulthood (2,223,385). Genetic lineage tracing results have demonstrated that zebrafish duct-associated progenitors can differentiate into all the pancreatic lineages (2), and this is consistent with observations that adult zebrafish exhibit a high capacity for regeneration following injury (247). Therefore, it is feasible and likely that the formation of complete, discrete pancreatic segments results from the differentiation of pancreas progenitors that had escaped long term effects of insulin deficiency during embryogenesis. This compensatory mechanism may resolve the exocrine pancreas insufficiency that would otherwise result from insulin deficiency during pancreas formation.

In addition to an the influence on the pancreatic progenitor pool, our results have also demonstrated that insulin signaling promotes pancreatic progenitor differentiation into exocrine lineages. Burlison et al. have shown that *Pdx1* and *Ptf1a* co-expressing cells exist only transiently in early pancreatic progenitor population (68). During differentiation, each these two transcription factors is selectively repressed in different cellular lineages: *Ptf1a* is repressed in endocrine and duct, while *Pdx1* is repressed in acinar cells (386). Here we have shown that insulin mediates acinar repression of *Pdx1*, and suggests that these insulin-deficient acinar cells may be trapped in a partially dedifferentiated state. Furthermore, we have revealed that knockdown of *insulin* results in formation of cells which retain both acinar and duct cell characteristics. All this

evidences supports the assertion that the exocrine pancreas differentiation program is highly dependent upon insulin signaling.

Besides early progenitor programming, our studies have also revealed a critical role for insulin signaling in distinguishing duct and acinar cell lineages during progenitor cell differentiation. Knockdown of *insulin* appears to produce cells in a confused meta-state, whereby they express both acinar and duct cell markers. It is likely that *insulin* regulates cell fate determination via key transcription factors. Previous studies have shown that specification of the pancreas relies on two key transcriptional factors, Pdx1 and Ptf1a (68). The Ptf1a transcriptional complex binds many acinar cell-specific gene promoters and acts as a master regulatory factor for acinar cell formation and function (387). Pdx1 is also expressed in the early pancreatic progenitor cells, and deletion of *Pdx1* in mice results in pancreatic agenesis (62). However, in the adult mammalian pancreas, Pdx1 is only strongly expressed in  $\beta$  cells; outside the islet, Pdx1 expression is weak in small pancreatic ducts and centroacinar cells, and acinar expression of Pdx1 is minimal (381,388). In our studies we found that the dynamic expression pattern of Pdx1 is conserved between mammalian and zebrafish adult pancreata. The repression of *Pdx1* in mature acinar cells is necessary for acinar cell functions (389), and persistent expression of *Pdx1* induce exocrine hypoplasia and a dramatic reduction in digestive enzyme expression (389). Nonetheless, although Pdx1 is not expressed in mature acinar cells, it is required for the formation of acinar cells during late pancreatic organogenesis (390). Using a Tet-off regulatory system, it is shown that deletion of *Pdx1* in later gestational stages blocked acinar cell formation and inhibited *Ptf1a* expression in acinar cells (390). Furthermore, activation of Pdx1 is observed in human and animal models pancreatitis, where it may be important for acinar cell regeneration (42,391). These findings contribute to a model in which Pdx1 is not completely silenced but rather dampened in mature acinar cells (392). Furthermore, these findings clearly demonstrate that the dynamic regulation of *Ptf1a* and *Pdx1* expression in acinar cells is crucial for acinar cell formation and regeneration.

The extracellular signals that regulate expression of these two key transcription factors in acinar cells are unknown. In this study, we have determined that insulin signaling may be an important extracellular cue that regulates *Pdx1* and *Ptf1a* expression

in pancreatic progenitor cells and acinar cells. The studies from Chapter Four showed that insulin deficiency during embryogenesis promotes *Pdx1* expression in the ventral endoderm progenitors. Moreover, our results here show that insulin signaling drives an increase in the quantity of *Ptf1a*-expressing cells. Altogether these data support the hypothesis that insulin represses *Pdx1*, but activates *Ptf1a* expression to promote acinar cell formation. It is well recognized from human clinical studies that T1DM is correlated with reduced exocrine pancreas size and function (393). In addition, insulin plays an important role as a trophic agent for acinar cells, which further supports the existence of an islet-acinar axis (137,394). In this study, we have determined that insulin signaling regulates both acinar cell formation and maturation, likely via the regulation of *Pdx1* and *Ptf1a* expression. Further understanding the molecular links between insulin signaling and the regulation of these key genes may spark new therapeutic strategies to confront T1DM-induced exocrine pancreas insufficiency.

This study also provides evidence that insulin signaling may contribute to the maintenance of acinar cell fate stability. The plasticity of acinar cells has been reported in many previous studies. In particular, it is reported that rat acinar cells can transdifferentiate into duct cells and re-activate *Pdx1* when cultured *in vitro*; this underscores the importance of the *in-vivo* micro-environment in maintaining acinar cell fate and *Pdx1* repression (395). Moreover, persistent expression of *Pdx1 in vivo*, induces acinar to duct transition (389), and duct-like cells or tubular complexes appear in both pancreatitis and pancreatic neoplasia in humans; this acinar to ductal metastasis underlies the initiation of acinar cell carcinoma (381,396,397). In this study, we demonstrate that insulin signaling plays an essential role in regulating the acinar and duct cell fates: knockdown of *insulin* results in abnormal duct-like acinar cell morphology, and inhibition of insulin signaling in acinar cells reactivates *Pdx1* expression, and results in the loss of acinar cell polarity.

Obesity and adult onset Type 2 diabetes are highly associated with development of acinar cell carcinoma (398,399), which suggests a link between insulin signaling and pancreatic cancer. In contrast to type 1 diabetes, type 2 results from relative insulin-deficiency due to unresponsiveness of target tissues to insulin, or “insulin resistance.” Although it was long held that insulin resistance was restricted to metabolically active

tissues like liver, skeletal muscle and adipocytes, recent work has broadened this view that “insulin resistance” in T2DM is a whole body physiological state associated with neurons, blood vessels, pancreatic endocrine cells and others (400-402). Although there is currently no evidence for acinar cell insulin resistance in T2DM, our studies suggest that reduced insulin signaling outputs in T2DM will promote the expression of Pdx1 in acinar cells and induce acinar to duct transdifferentiation, and this may then contribute to cancer development.

Besides acinar to duct transformation, acinar cells can be transformed into  $\beta$  cells under some experimental conditions. Zhou et al. showed that the transfection of three transcription factors: Pdx1, Ngn3, and Mafa, is sufficient to drive acinar cell conversion into  $\beta$  cells (182). In addition, acinar to duct cell conversion can be achieved via repressing *Ptf1a* activity (183). However, in this study, we did not detect acinar conversion after loss of insulin signaling. This indicates that Pdx1 misexpression alone is not sufficient to surmount the epigenetic barriers between acinar cells and endocrine cells, and is consistent with the findings of Zhou et al. (180), and that Ngn3 is required to initiate the pancreatic endocrine program (403). Moreover, Ngn3 is ectopically expressed in exocrine pancreas following partial duct ligation (PDL), an inflammation-associated pancreatic injury model (176,404). Furthermore, acinar to endocrine/ $\beta$ -cell conversion is triggered by PDL combined with pharmacological elimination of pre-existing  $\beta$ -cells (405). In accord with all these studies, our results have shown that insulin signaling also plays a critical role in acinar cell fate regulation. Therefore, it may be productive to investigate combinations of insulin suppression and Ngn3 induction in driving the transformation of acinar cells into  $\beta$  cell for the treatment of both T1DM and T2DM.

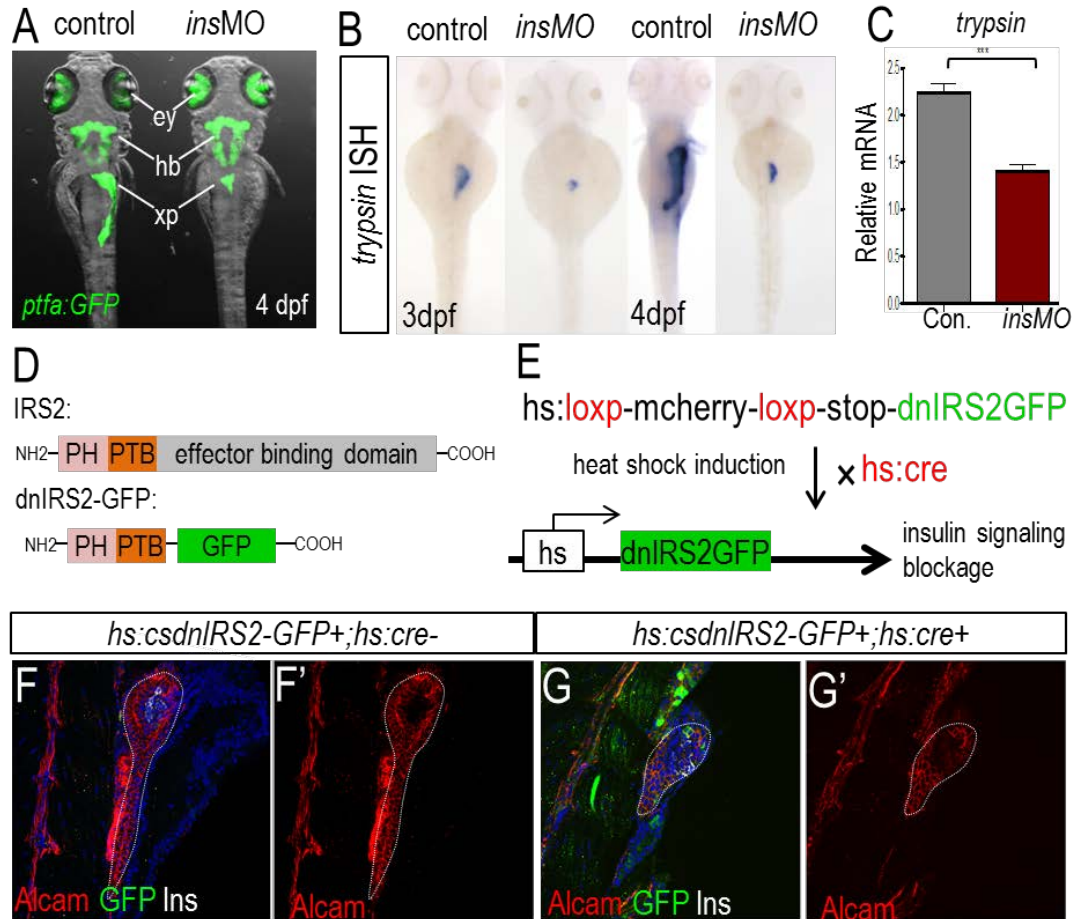


Figure 5-1. Blocking insulin signaling impairs exocrine pancreas development. (A) 4 dpf *Tg(ptf1a:GFP)* control embryos and embryos injected with *insaMO* at one cell stage. ey:eye; hb: hindbrain; xp: exocrine pancreas. (B) In situ hybridization of *trypsin* expression in 3dpf and 4dpf control and *insaMO* embryos. (C) real time PCR of trypsin mRNA expression in 4dpf control and *insaMO* embryos, n=3. (D) Illustration of c-terminal deletion form of dnIRS2-GFP construct. (E) Illustration of blocking insulin signaling using *Tg(hs:CSdnIRS2)* and *Tg(hs:cre)* system. (F-G) Confocal projection of 84hpf *Tg(hs:CSdnIRS2-GFP)* and *Tg(hs:CSdnIRS2-GFP); Tg(hs:cre)* pancreas stained for Alcam(red), GFP(green) and Insulin (white). Embryos were heat shock in 10hpf, 24hpf, 36hpf, 48hpf, 56hpf and 72hpf to induce *dnIRS2-GFP* expression. Student t-test was used in C for statistical analysis.

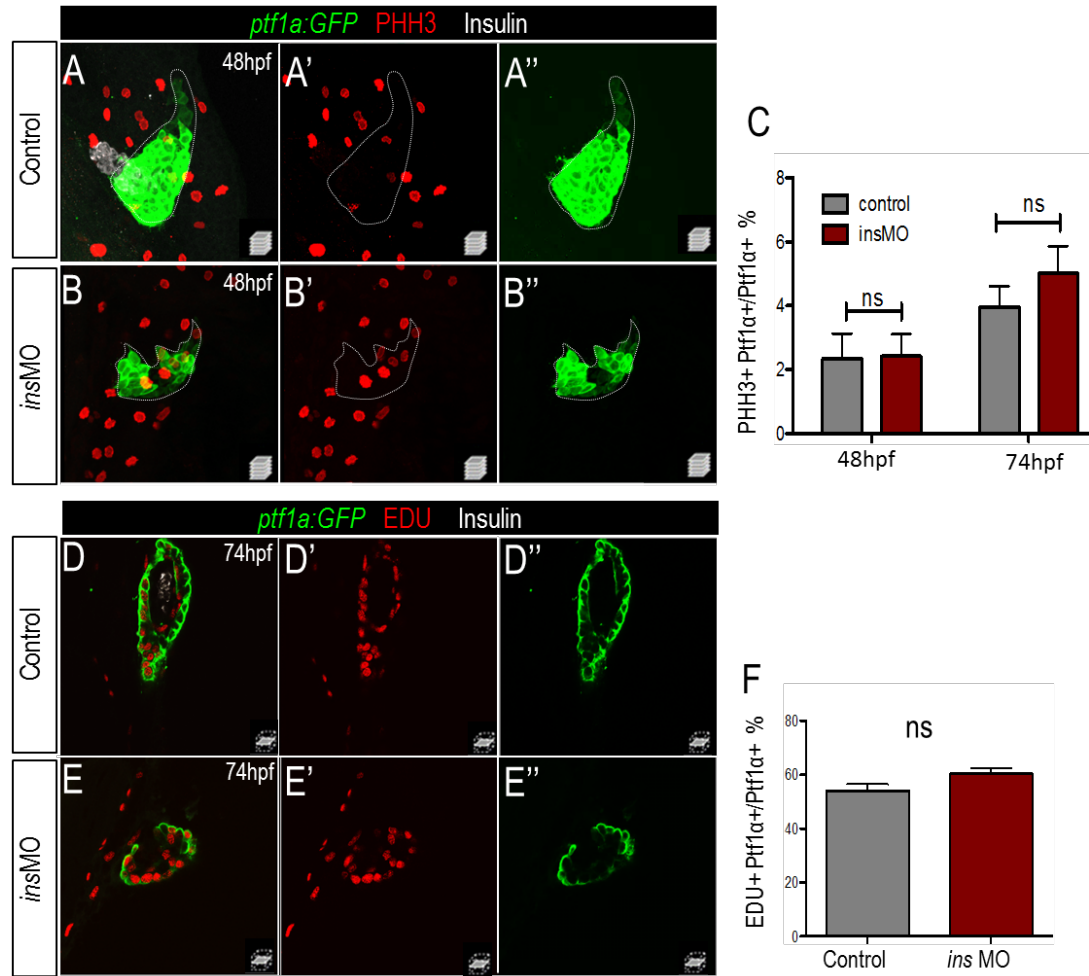


Figure 5-2. Knockdown insulin does not decrease proliferation of exocrine pancreas. (A-B) Confocal projection of 48 hpf *Tg(ptf1a:GFP)* control and *insaMO* injected exocrine pancreas stained for GFP (green), Insulin (white) and PHH3 (red). (C) Quantification of PHH3+ *ptf1a*+ cell percentage in 48hpf and 74hpf control and *insaMo* embryos. (D-E) Confocal plane of 74 hpf *Tg(ptf1a:GFP)* control and *insaMO* injected exocrine pancreas stained for GFP(green), Insulin(white) and EDU(red). EDU solution was infused into embryos by injecting into pericardia sac 1 hour before fixation. (F) Quantification of EDU+ *ptf1a*+ cell percentage in 74 hpf control and *insaMO* embryos. Two way Anova was used in C and student t-test used in F for statistical analysis.



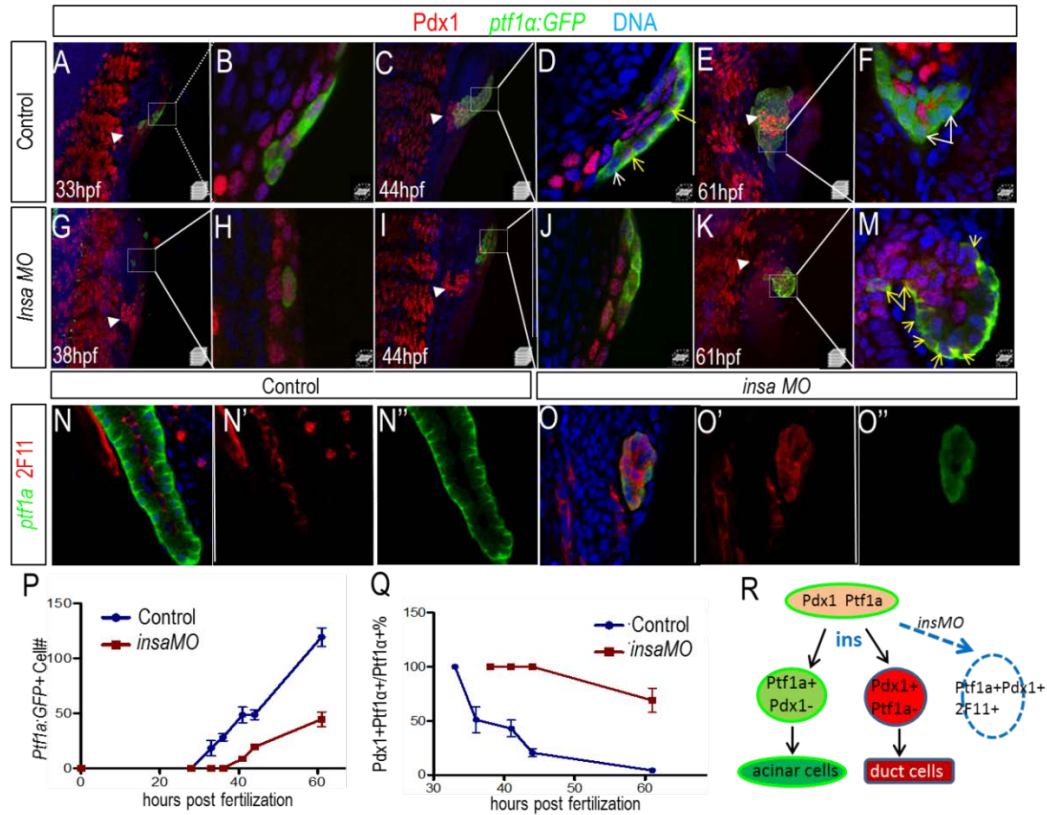


Figure 5-3. Insulin knockdown delays *ptfla* initiation and impairs acinar and duct cell specification. (A-M) Confocal projections and planes of *Tg(ptfla:GFP)* in control and *insaMO* pancreatic endoderm stained for Pdx1 (red) and GFP (green). White arrow head indicates the principal islet. White arrow indicates *ptfla*<sup>+</sup> Pdx1<sup>-</sup> cells. Yellow arrow indicates remaining of *ptfla*<sup>+</sup> Pdx1<sup>+</sup> cells in 61hpf *insaMO* embryos. (N-O) Confocal planes of *Tg(ptfla:GFP)* in control and *insaMO* pancreatic tail stained with duct marker 2F11 (red). Noted that in control pancreas, 2F11<sup>+</sup> cells are *ptfla: GFP*<sup>-</sup> but in *insaMO* pancreas 2F11<sup>+</sup> cells are *ptfla: GFP*<sup>+</sup>. (P) Quantification of total *ptfla*<sup>+</sup> cell number in control and *insaMO* injected embryos. (Q) Quantification of Pdx1+ *ptfla*<sup>+</sup> cell percentage in control and *insaMO* injected embryos. (R) Illustration of the model of insulin regulates exocrine pancreas differentiation. Insulin signal is required for Pdx1+Ptf1a<sup>+</sup> pancreatic progenitors differentiate into both Pdx1+Ptf1a<sup>-</sup> duct progenitor lineage and Pdx1-Ptf1a<sup>+</sup> acinar progenitor lineage. Knockdown of *insulin* signal interrupts the progenitor cell differentiate appropriately into acinar and duct cell lineage and induces the formation of cells have mixed acinar and duct cellular characters.

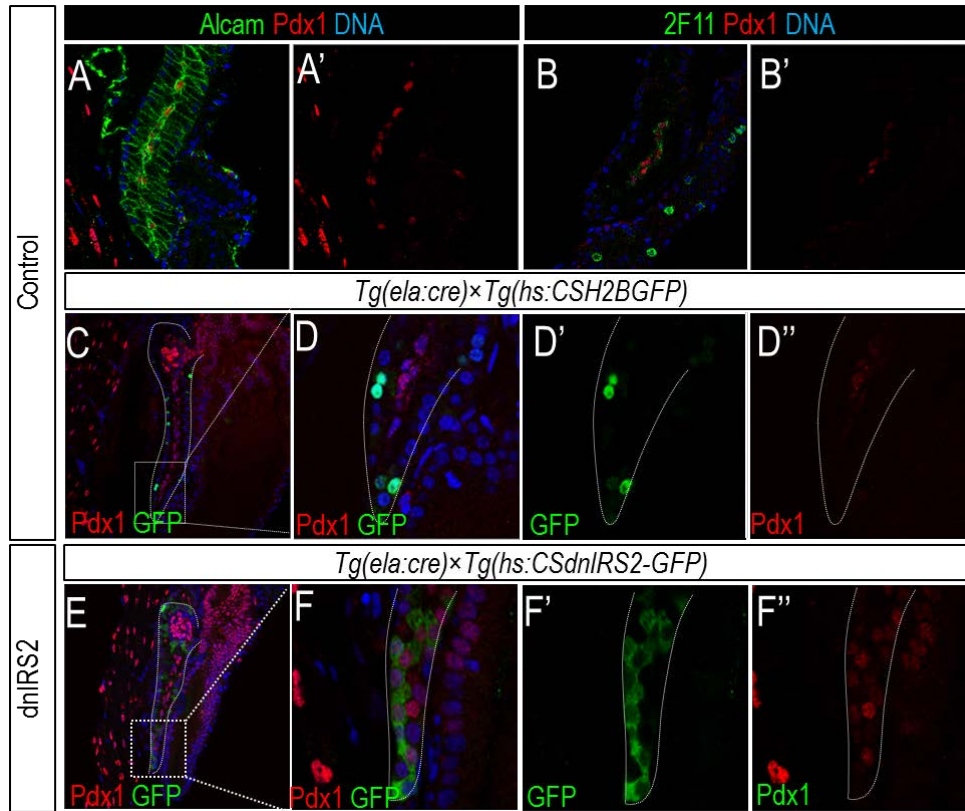


Figure 5-4. Inhibition of insulin signal in acinar cells re-activates Pdx1 expression. (A) Confocal plane of 84hpf pancreatic tail stained for Alcam (green) and Pdx1 (red). It is noted that Pdx1+ duct cells exhibit distinct cell morphology compared with Pdx1- acinar cells. (B) Confocal plane of 84hpf pancreatic tips stained for 2F11 (green) and Pdx1 (red) showing Pdx1 was limited in 2F11+ pancreatic duct cells. (C) Confocal plane of 84hpf *Tg(hs:CSH2B-GFP); Tg(ela:cre)* pancreas stained for GFP(green) and Pdx1(red). Embryos were heat shocked in 48hpf, 56hpf and 72hpf to induce H2B-GFP expression in acinar cells. (D) Zoom in of pancreatic tips in E showing Pdx1 expression was limited in pancreatic ducts and no H2B-GFP+ cells express Pdx1. (E) Confocal plane of 84hpf *Tg(hs:CSdnIRS2-GFP); Tg(ela:cre)* pancreas stained for GFP(green) and Pdx1(red). embryos were heat shocked in 48hpf, 56hpf and 72hpf to induce dnIRS2-GFP expression in acinar cells. (F) Zoom in image of pancreatic tips in C showing the expression of Pdx1 in dnIRS2-GFP expression acinar cells.

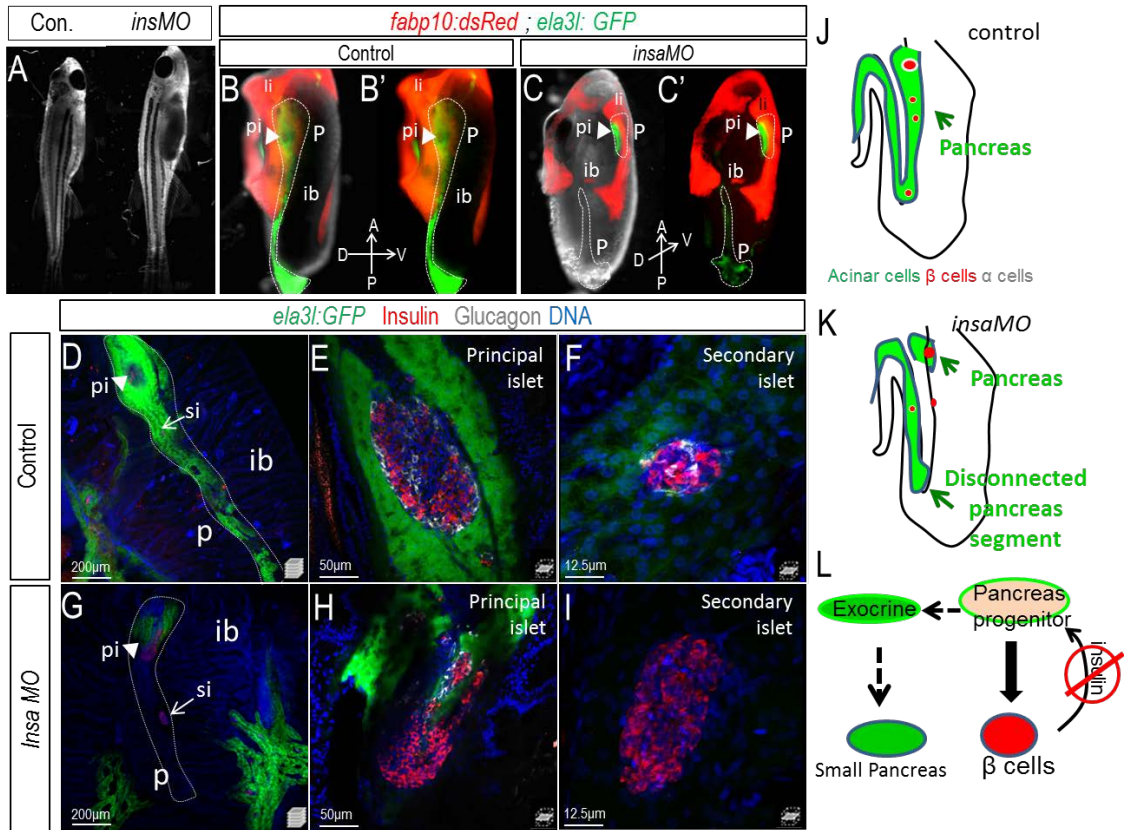


Figure 5-5. Embryonic exocrine pancreas deficiency influences adult exocrine pancreas. (A) 2 month old control and *insaMO* injected fish. (B-C) Endoderm of 2 month old control and *insaMO* injected fish marked by *Tg(fabp10:dsRed); Tg(ela3l:GFP)*. Pi: principal islet; ib: intestine bulb; p: pancreas; li: liver. (D, G) Confocal projections of 2 month old control and *insaMO* injected endoderm marked by *Tg(ela3l:GFP)* stained for Insulin(red) and Glucagon(white). Pi: principal islet; si: secondary islet; ib: intestine bulb; p: pancreas. Arrow heads showing the principal islet and arrow showing the secondary islet. (E-H) Confocal projections of 2 month old control and *insaMO* injected principal islet stained for Insulin (red) and Glucagon (white). (F-I) Confocal projections of 2 month old control and *insaMO* injected secondary islet indicated by arrows in D and G which is stained for Insulin (red) and Glucagon (white). It is noted that there is no Glucagon+ cells in *insaMO* injected secondary islet. (J-K) Illustration of adult pancreas in control and *insaMO* injected fish. (L) Illustration of the model for insulin regulates pancreatic development. Without receiving feedback insulin signal from  $\beta$  cells, pancreatic progenitor cells differentiate early into  $\beta$  cells. The reduced progenitor pool and impaired exocrine pancreas differentiation program will result in the reduced exocrine mass.

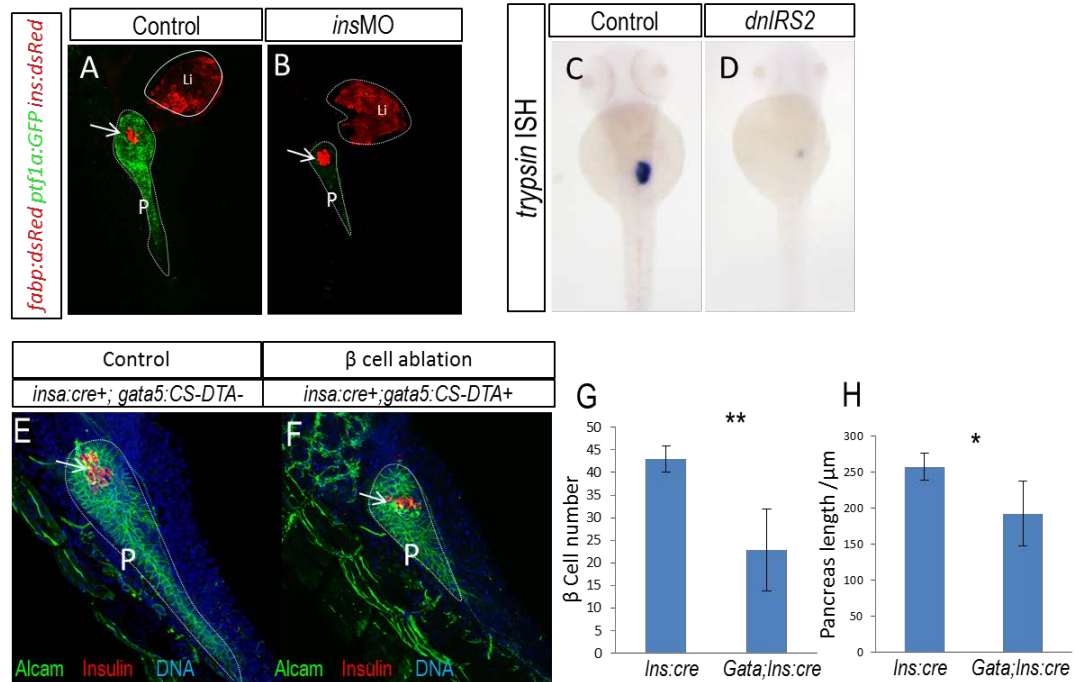


Figure 5-6. Inhibition of Insulin signal impairs exocrine pancreas development. (A-B) Confocal projection of both control and *insaMO* injected endoderm in 3 dpf *Tg(fabp10:dsRed); Tg(ptf1a:GFP); Tg(ins:dsRed)* embryos. White arrows indicate the principal islet. li:liver; p: pancreas. (C-D) In Situ Hybridization showing the expression of *trypsin* in both control and *dnIRS2-GFP* mRNA injected embryos at 54 hpf. (E-F) Confocal projections of control and  $\beta$  cell ablation endoderm stained with Alcarn(green) and Insulin(red) at 72 hpf. Control:  $Tg(ins:cre)^+ Tg(gata5:loxp-mcherry-loxp-DTA)^-$ ;  $\beta$  cell ablation:  $Tg(ins:cre)^+ Tg(gata5:loxp-mcherry-loxp-DTA)^+$ . (G) Quantification of  $\beta$  cell number in both 72 hpf control and  $\beta$  cell ablating embryos. (H) Quantification of pancreatic length marked by Alcarn in both 72 hpf control and  $\beta$  cell ablating embryos. Student T-tests were used in I, L, M for statistical analysis.

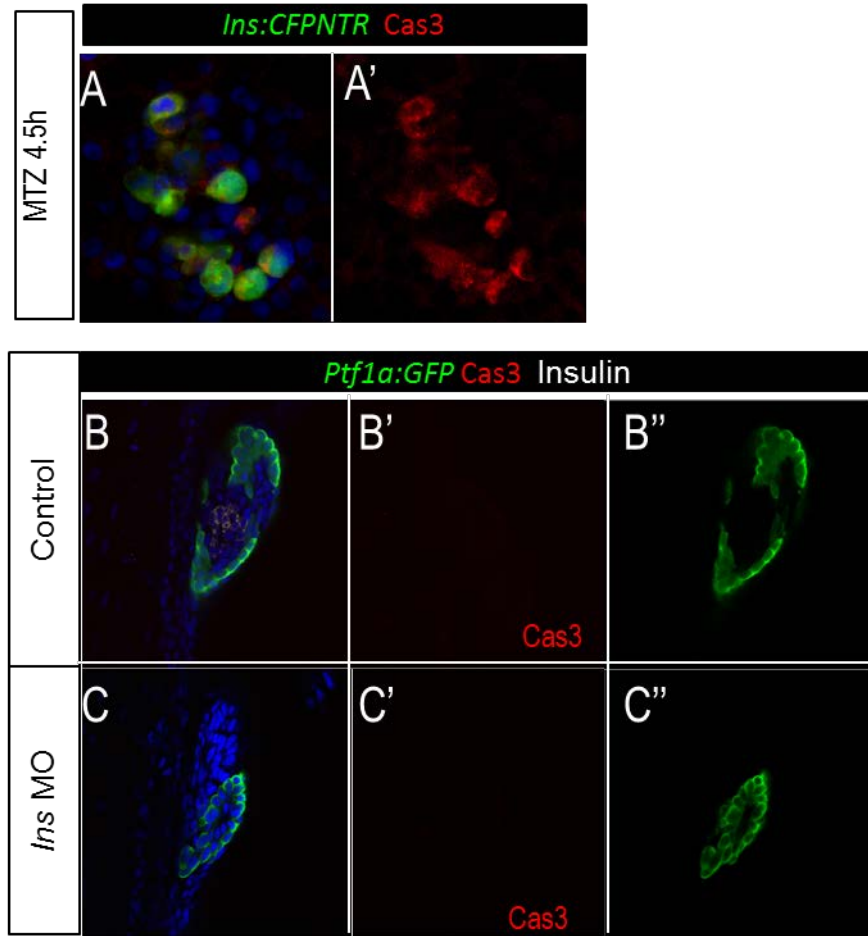


Figure 5-7. Knockdown of *insulin* does not increases exocrine pancreas apoptosis. (A) Confocal plane image of 3 dpf islet in *Tg(ins:CFP-NTR)* treated with MTZ for 4.5 hours stained with CFP(green) and Cleaved-Caspase 3(red). (B-C) Confocal plane of 54 hpf pancreas in *Tg(ptf1a:GFP)* control and *insaMO* injected embryos stained with Insulin(white) and Cleaved-Caspase3(red).



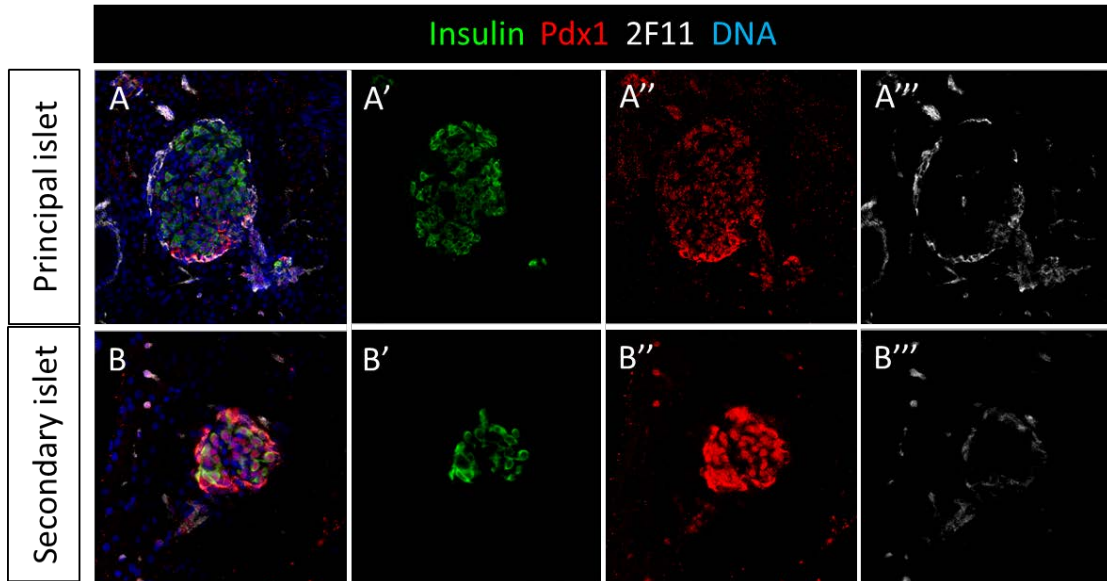


Figure 5-8. Pdx1 expression is limited to adult zebrafish islet  $\beta$  cells and duct cells. (A-B) Confocal plane of 2 month old adult zebrafish principal islet (A) and secondary islet (B) stained with Insulin (green), Pdx1 (red) and 2F11 (white). It is noted that Pdx1 expression is limited to Insulin+ or 2F11+ cells in the pancreas.

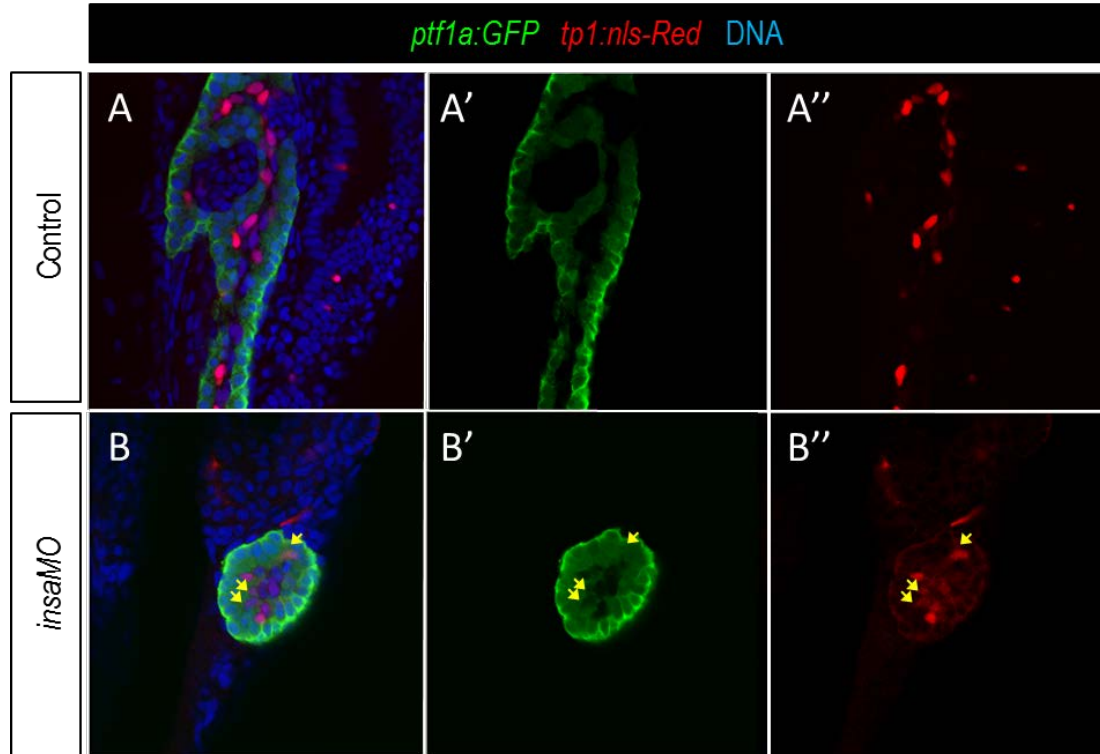


Figure 5-9. Knockdown of *insulin* induces the formation cells co-express both acinar and duct cell markers. (A-B) Confocal plane of 72 hpf *Tg(tp1:nls-Red); Tg(ptf1a:GFP)* control (A) and *insaMO*-injected zebrafish embryos. Yellow arrows in B indicate *tp1:nls-Red*<sup>+</sup> *ptf1a:GFP*<sup>+</sup> cells. It is noted that no double positive cells can be detected in A.



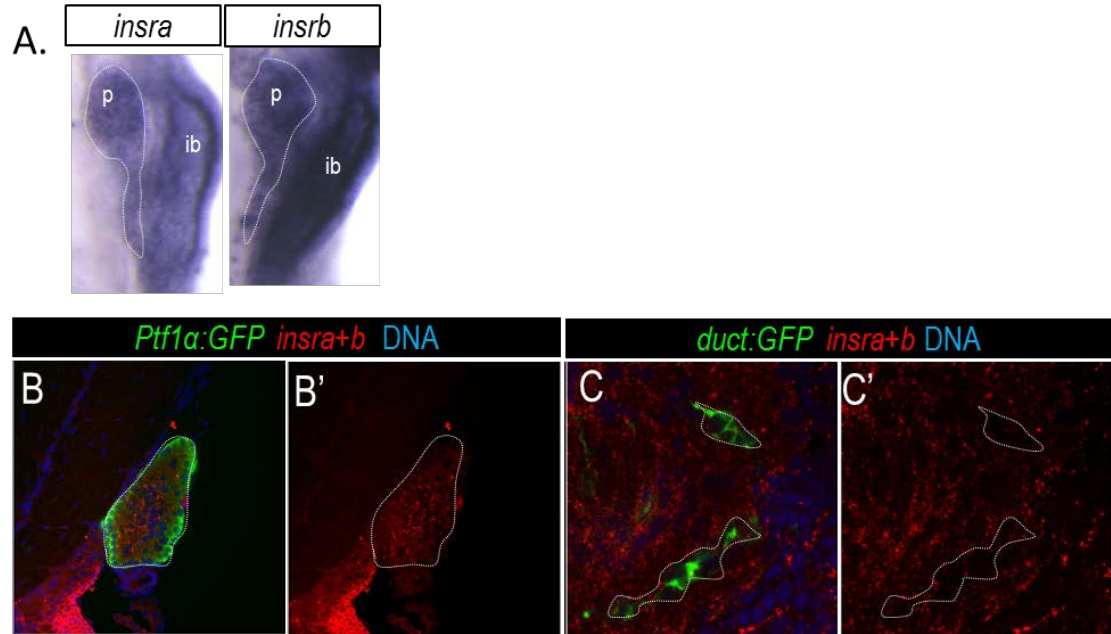


Figure 5-10. Expression of *insulin receptors* in acinar cells. (A) The expression of *insulin receptor a* (*insra*, A) and *insulin receptor b* (*insrb*, B) at 3 dpf embryonic endoderm regions using In Situ Hybridization. p: pancreas, ib: intestine bulb. (B) Fluorescence In Situ Hybridization detecting the expression insulin receptors (red) in 3 dpf *Tg(ptf1a:GFP)* embryos. (C) Fluorescence In Situ Hybridization detecting the expression insulin receptors (red) in 3 dpf *Tg(nkx2.2:GFP)* embryos. In both B and C, *insr* probe was designed to bind at homologous region of both *insra* and *insrb*. It is noted that insulin receptor is strongly expressed in *ptf1a: GFP*<sup>+</sup> acinar cells but weak or not expressed in *nkx2.2: GFP*<sup>+</sup> pancreatic duct cells.

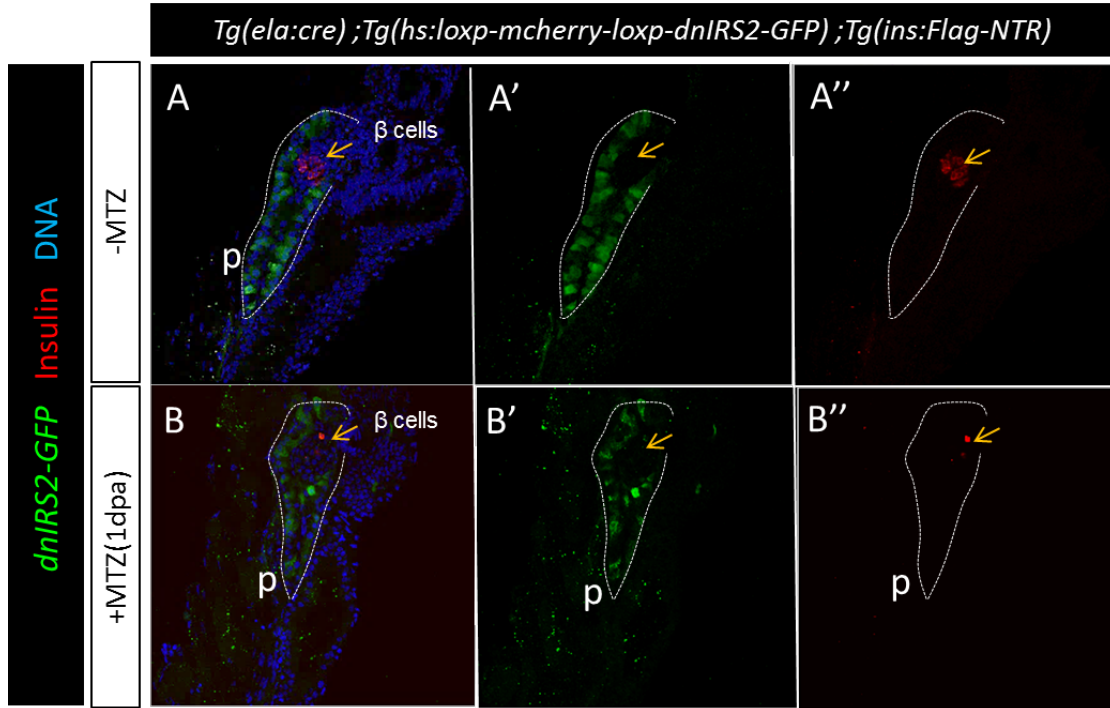


Figure 5-11. Block insulin signaling in acinar cells does not convert acinar cell into  $\beta$  cells. (A) Confocal image of 4 dpf *Tg(ela3l:cre) Tg(hs:loxp-mcherry-loxp-dnIRS2-GFP)* embryos stained with GFP(green) and Insulin(red). (B) Confocal image of 4 dpf *Tg(ela3l:cre) Tg(hs:loxp-mcherry-loxp-dnIRS2-GFP)Tg(ins:Flag-NTR)* embryos with MTZ treatment from 2 dpf to 3 dpf to ablate  $\beta$  cells stained with GFP(green) and Insulin(red). It is noted that no  $\text{dnIRS2-GFP}^+ \text{Insulin}^+$  cells were detected in A or B.

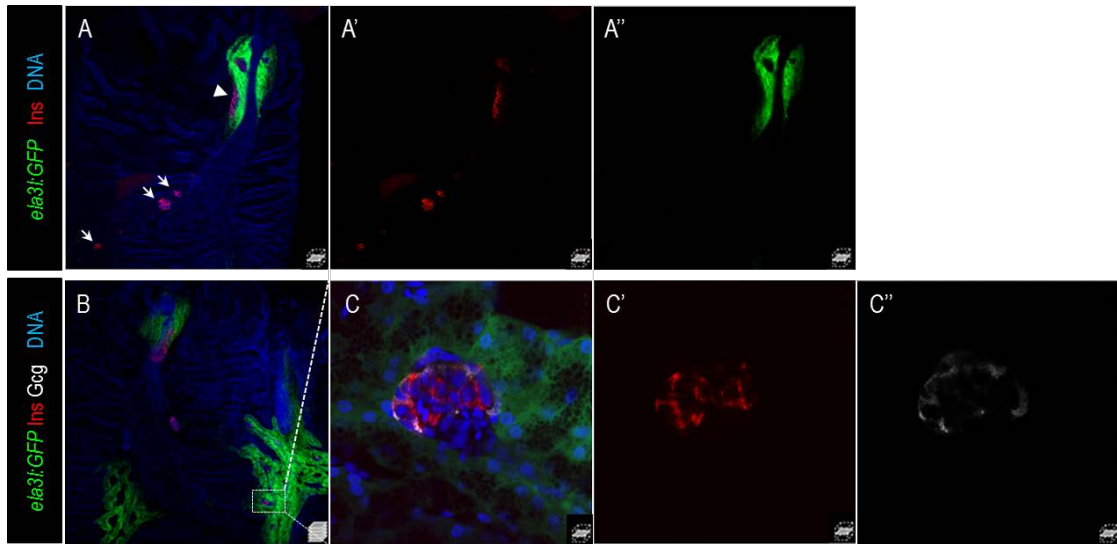


Figure 5-12. Secondary islets in extra-pancreatic segments exhibit normal morphology in 2 month old *insaMO* injected fish. (A) Confocal plane of 2 month old *insaMO* injected *Tg(ela3l:GFP)* stained for Insulin(Red). Arrow head indicate the location of principal islet and arrows indicate the “naked” secondary islet. (B) Confocal projection of 2 month old *insaMO* injected *Tg(ela3l:GFP)* stained for Insulin(red) and Glucagon(white). (C) Confocal plane of secondary islet located in *Tg(ela3l:GFP)* extra-pancreas segments stained for Insulin(red) and Glucagon(white). It is noted that there is normal glucagon expression in these islets.

## **CHAPTER SIX: Summary and future directions**

### **VI.A. Summary and Implications**

Although the roles of pancreatic hormones in regulating metabolic homeostasis are well understood, the influence of pancreatic hormone on pancreatic endocrine cell formation during both developmental and tissue regeneration phases are poorly investigated. In this dissertation, I have used various genetic and pharmaceutical approaches to identify the novel roles of pancreatic hormones in regulating pancreatic endocrine homeostasis. Interestingly, I also showed that the exocrine pancreas component is strongly influenced by insulin signaling during development. In particular, we have shown for the first time that the balance between insulin and glucagon signaling is required to maintain endocrine cell fate stability and pancreatic progenitor self-renewal and differentiation. Therefore, fully understanding the roles of pancreatic hormones in regulating the development and differentiation of the pancreas not only will shine light on understanding the integrative roles of pancreatic cells types in the development process but also brings insight into the pathology of diseases like Diabetes. Moreover, understanding the roles of pancreatic hormones in pancreatic endocrine homeostasis will lead to the development of new therapeutic targets to promote regeneration of new  $\beta$  cells, which will ultimately bring hope to the cure of Diabetes.



roles in pancreatic progenitor domain initiation (92,93). The mass of this progenitor pool was shown by previous studies to determine the whole pancreatic organ size (115). However, the regulation of pancreatic progenitor self-renewal and differentiation is unclear. Moreover, through the development process, the pancreatic progenitor pool will ultimately differentiate into different endocrine and exocrine cell lineages. Although a variety of growth factors and cytokines secreted from surrounding mesenchyme and pancreatic ductal epithelium have been proposed to play roles in regulating pancreatic progenitor differentiation (94,98,99,406,407), it is wholly unknown how the organism achieves the balance between endocrine and exocrine ratio as well as cell quantity control for different endocrine cell types through the development process. In this dissertation, through investigating the complicated roles of *insulin* and *glucagon* genes in pancreas endocrine and exocrine formation, I have revealed a feedback regulatory system that functions during pancreas development at different levels: from islet cells to pancreatic progenitors; from pancreatic endocrine cells to exocrine cells; interaction between  $\alpha$  cells and  $\beta$  cells. Moreover, the pancreatic hormones can influence the pancreas development process via integrating the metabolic cues such as glucose regulation. The stem cell transplantation I have developed here can contribute to the understanding of circulating factors derived from pancreatic hormones' effects during embryonic development. Moreover, understanding the extracellular signaling control of pancreas development regulating pancreatic progenitor differentiation and cell fate specification can help determine the targets which can stimulate *in vitro*  $\beta$  cell differentiation from stem cells or neogenesis from adult pancreatic progenitor cells.

It is well appreciated that gestational diabetes is strongly associated with the development of diabetes in offspring in later life (408,409). Interestingly, another set of studies have shown that maternal starvation during pregnancy is also strongly associated with the development of diabetes in offspring in later life (410-412). During development, the embryo exchanges nutrients with the mother and embryonic pancreas hormone secretion is tightly associated with the environmental nutrient (413,414). The conclusions from this dissertation indicate that inappropriate pancreatic hormone secretion resulting from the abnormal nutrient state will alter the embryonic pancreas

development program, which could ultimately contribute development of diabetes later in life. Future studies using mammalian models are required for testing such hypothesis.

### **$\beta$ Cell regeneration for Diabetes treatment**

From the systemic view, there are two driving forces to create the need for  $\beta$  cell formation: impaired  $\beta$  cell mass and insufficient  $\beta$  cell function. The latter can be mainly characterized by insufficient insulin signaling. Considering the cellular origins,  $\beta$  cells can regenerate from self-replication and neogenesis from non- $\beta$  cells. Although the mechanisms involved in the regulation of  $\beta$  cell self-replication induced regeneration is highly investigated (22,162,200,290,415), the cell signaling that regulates  $\beta$  cell regeneration from neogenesis is relatively unknown. In this dissertation, I have investigated the process of  $\beta$  cell neogenesis following  $\beta$  cell injury and insulin signaling blockage. Specifically, I have revealed in zebrafish models that  $\beta$  cells mainly regenerate from two cellular sources: pancreatic progenitor differentiation and  $\alpha$  cell transdifferentiation. Importantly, I have found that the insulin and glucagon hormones play critical roles in controlling  $\beta$  cell regeneration from both cellular sources. Interestingly, similar to metabolic regulation, insulin and glucagon hormones also appear to play counter-balancing roles in directing  $\beta$  cell formation and endocrine cell fate specification. Revealed by this dissertation, glucagon signaling can promote  $\beta$  cell formation via increasing progenitor cell differentiation and destabilizing  $\alpha$  cell fates to induce  $\alpha$  to  $\beta$  cell transdifferentiation. On the other hand, insulin signaling can inhibit the early differentiation of pancreatic progenitors and stabilize  $\alpha$  cell fate. This indicates that under normal conditions, an imbalanced glucagon and insulin ratio can be corrected by the feedback mechanisms via adjusting  $\alpha$  and  $\beta$  cell ratios.

Therefore, it will be interesting to understand whether these feedback correction mechanisms are impaired under pathological conditions like diabetes. Moreover, further understanding the downstream effectors which control the pancreatic hormonal feedback mechanisms will help with the design of novel drug targets for promoting *in vivo*  $\beta$  cell regeneration from either  $\alpha$  cell transdifferentiation or pancreatic progenitor cell differentiation. Furthermore, understanding the effects of pancreatic hormone in  $\beta$  cell

formation will also contribute to the design of a rationale protocol to achieve a higher yield for *in vitro* differentiation from stem cells toward  $\beta$  cells within shorter time. However, compared with *in vitro*  $\beta$  cell regeneration approaches, *in situ* regeneration of  $\beta$  cells from endogenous sources would circumvent many of the obstacles encountered by surgical restoration of  $\beta$  cell mass via transplantation. The studies presented here have defined tunable endogenous cellular sources for  $\beta$  cell regenerating which can be regulated by physiological hormones. Therefore, it provides valuable directions for future studies in the field of *in vivo*  $\beta$  cell regeneration based diabetes therapeutic design.

### **Exocrine pancreas insufficiency and mutagenesis**

As an integrated organ, the endocrine and exocrine pancreas serves different but highly connected functions to achieve the homeostasis for metabolism. Although it has long been appreciated that the function of the exocrine pancreas is highly influenced by hormones secreted from islet cells (137), it is not known whether this islet-exocrine axis exists during embryonic development. The studies in this dissertation have revealed the unique developmental roles of insulin signaling in regulating the differentiation and maturation of exocrine acinar cells. Moreover, we also found that insulin signaling plays important roles in stabilizing acinar cell fate. These findings contribute to the understanding of research areas, such as diabetes associated exocrine pancreatic disease. For example, it is well known that early on-set T1DM often results in exocrine pancreatic insufficiency (373,416,417). Meanwhile T2DM is found to be strongly associated with pancreatic cancer (399). Although the mechanisms underlying pancreatic cancer are not completely understood, more and more evidence indicates that acinar to duct metaplasia is highly involved in the early pancreatic cancer progression (396,397). Therefore, it will be interesting to test whether the “insulin resistant” stage in acinar cells will induce the acinar cell fate changes, which will ultimately promote the cancer development process in T2DM and reveal novel drug targets for pancreatic cancer treatments.



## VI.B. Future Directions

### The mechanisms of pancreatic hormone regulated pancreas development

I have demonstrated in this dissertation that insulin and glucagon signaling will influence the cell fate decisions during early pancreatic progenitor differentiation. This feedback regulation mechanism is critical to regulate  $\alpha$  and  $\beta$  cell number during development. Although glucagon increased progenitor cell differentiation may be indirectly mediated via glucose, the insulin signaling appears to directly act on early pancreatic progenitors. Therefore, it is necessary to further dissect the complicated interactions between glucagon, glucose and insulin in regulating pancreatic progenitor differentiation.

(1) Examine the genetic profile during the differentiation process from pancreatic progenitor into  $\alpha$  and  $\beta$  cell lineages. We will first use transgenic zebrafish embryos *Tg(gcga:GFP); Tg(ins:dsRed); Tg(sst2:mKate2); Tg(neurod:BFP)* and perform cell sorting to isolate *neurod:BFP<sup>+</sup>* (undifferentiated endocrine progenitor cells), *neurod:BFP<sup>+</sup>ins:dsRed<sup>+</sup>* ( $\beta$  cells), *neurod:BFP<sup>+</sup>gcga:GFP<sup>+</sup>* ( $\alpha$  cells) and *neurod:BFP<sup>+</sup>sst2:mKate2<sup>+</sup>* ( $\delta$  cells). Genetic sequencing will then be performed on these cell populations. These data will be further used to specifically analyze the signals associated with the glucagon, *insulin* and glucose metabolism pathways. The signaling pathway activation can then be analyzed together with the innate genetic transcription factors to give the link between the signal and gene transcription. This approach can also be used to define signal targets involved in progenitor differentiation and those that discern the various endocrine cell lineages.

(2) In vitro cell culture system to analyze the downstream signaling involved in pancreatic hormone regulated pancreas progenitor differentiation. Primary zebrafish endocrine progenitor cells can be obtained by cell sorting (as indicated above) and then cultured *in vitro*. Following treatment with glucagon/insulin/glucose signaling activators and inhibitors, real-time RT-PCR can be used to analyze the transcription factors involved in downstream cell signaling and cell fate determination. For example, from the studies in this thesis, insulin signaling can regulate pancreatic progenitor differentiation via manipulating the expression of two key transcription factors *pdx1* and *ptfla*.

Furthermore, the differentiation of progenitor cell is highly depended on Notch signaling pathways. Using this approach, we can identify tested the following hypothesis that insulin regulates the expression of *pdx1* and *ptf1a* via manipulate downstream Notch target genes such as *hes1*. Similarly, we can test how pancreatic hormones influence the expression of key transcriptions factors involved in  $\alpha$  and  $\beta$  cell fate determination such as *arx* and *mafa*. Besides zebrafish primary endocrine cell culture, the mammalian endoderm progenitors, which can be differentiated from embryonic stem cells, can also be used to further verify the signals in mammalian systems.

In addition to analyzing the target genes, we can also use the large chemical screening approach to identify the chemical compounds which can stimulate progenitor differentiation. The undifferentiated pancreatic progenitor cells (*neurod:BFP*<sup>+</sup>, *ins:dsRed*<sup>-</sup>; *gcga:GFP*; *sst2:mKate2*<sup>-</sup>) can be isolated from *Tg(gcga:GFP)*; *Tg(ins:dsRed)*; *Tg(sst2:mKate2)*; *Tg(neurod:BFP)* as indicated above. If these progenitor cells differentiate into  $\beta$  cells, it will turn on the *insulin* promoter and start to express red fluorescent protein. If  $\alpha$  cell fate is activated under certain stimulations, the *glucagon* promoter will be turned on and these cells will express green fluorescent protein. This system can then be used to screen chemical compounds from a compound library which contains the chemicals involved in pancreatic hormones and cell metabolism pathways. Using this high-throughput screening can help identify novel targets to stimulate pancreatic progenitor differentiation.

(3) In vivo genetic approach to analyze the effects of glucagon/glucose/insulin on pancreatic progenitor differentiation. In addition to providing valuable insight into the control of pancreas development and regeneration, my dissertation research has generated multiple genetic tools which can be used to further investigate downstream signaling pathways involved in pancreatic hormone regulated cell differentiation *in vivo*. For example, we can use this approach to selectively activate the glucagon downstream signaling in progenitors to investigate the direct effects of glucagon stimulation on pancreatic progenitors. One signaling pathway involved in glucagon regulation is via stimulation of cAMP dependent-protein kinase A (PKA) (118). Binding of cAMP by the regulatory subunits of PKA results in their dissociation from the catalytic subunits and activation of the enzyme (418). Previous studies have shown that constitutive activation

of PKA can be achieved via overexpression of catalytic subunit of PKA (419,420). Therefore, we can investigate whether over expression constitutive activated PKA (aPKA) in endocrine progenitor can mimic glucagon effect and drive progenitor differentiation into  $\beta$  cells. For doing this, we can create the following transgenic fish *Tg(sox17: loxp-mcherry-loxp-aPKA); Tg(neurod1:cre)*. The caveat here is that *neurod* is not only expressed in pancreatic endocrine progenitors, but is also found in differentiated pancreatic endocrine cells as well as enteroendocrine cells. However, the genetic sequencing experiments proposed above may provide new targets to define the zebrafish pancreas endocrine progenitor specific genes. Besides the traditional transgenic approach, the current advance in Cas9-mediated genomic editing also makes it possible to create tissue specific knockout in zebrafish in a short time period and this knockout approach can also be used to verify the results obtained from the *in vitro* findings (421).

(4) Exploring pancreatic hormone-mediated progenitor cell differentiation using a cell transplantation system. In this dissertation, I have developed a novel model in which blastula stem cells are transplanted into zebrafish host larvae in order to investigate the effects of insulin signaling on pancreas progenitor differentiation. Using this approach, we have shown that there are complicated interactions between transplanted stem cells and the host system. The relatively easy genetic manipulation in zebrafish donor and host embryos and the transparency of zebrafish makes it a powerful model to further explore the stem cell-host interactions. For example, increased glucagon signaling in the host larvae following  $\beta$  cell ablation may be a cue to influence transplanted stem cell differentiation. To test this hypothesis, future studies may include transplantation of *dnIRS2*-overexpression blastula stem cells into  $\beta$  cell ablated larvae in the background of *gcgaMO* or *gcgr/glp1r* mutants. Furthermore, our findings provide evidence that transplanted “insulin resistant” blastula stem cells can secrete certain factors that facilitate host  $\beta$  cell regeneration. In the future, we will isolate the differentiated blastula stem cell after transplantation into the host and perform a large scale genetic sequencing study to determine potential targets that stimulate  $\beta$  cell regeneration.

(5) Exploring the mechanisms by which pancreatic hormones maintain acinar cell fate. In this dissertation, I showed that inhibition of insulin signaling in acinar cells can activate Pdx1 expression. The next question is whether these Pdx1+ acinar cells can be

further directed and differentiated into  $\beta$  cells. Previous studies show the potential of acinar cell transdifferentiation via repressing *ptf1a* or virus induced transfection of three key  $\beta$  cell transcription factor: Pdx1, Mafa and Ngn3. The expression of Ngn3 can be activated in partial duct ligation mouse models, which is proposed to be induced by immune factors. Thus, the following hypothesis that can be tested in the future is the following: reprogramming of acinar cells can be achieved via combining insulin signaling suppression and immune factors stimulation. It will be also interesting to understand how inhibition of insulin signaling alleviates the suppression mechanism of Pdx1 in acinar cells. Although it is proposed that in differentiated  $\beta$  cells, insulin activates Pdx1 expression via repressing Foxo activity, our studies indicate that in certain cell types, such as endoderm progenitor cell and acinar cells, insulin signaling can suppress Pdx1 expression, indicating that a link other than Foxo may exist between insulin and Pdx1. Finding such a link will increase our understanding of the complex role that insulin plays in regulating pancreas development and cell fates. Besides acinar to  $\beta$  cell transformation, another attractive direction of investigation is to pursue the possibility that insulin signaling promotes pancreatic cancer metastasis. To test that idea, we can create a pancreatic cancer model by over-express the oncogene, *k-ras*, in acinar cells using the elastase promoter and examine how an insulin resistant state influences cancer progress.

### **The mechanism by which pancreatic hormones regulate $\alpha$ cell fate and stability**

In this dissertation, I have shown that pancreatic hormones like insulin and glucagon not only play important roles in regulating pancreatic progenitor differentiation, but also govern  $\alpha$  cell fate and stability. More importantly, we have shown that  $\alpha$  cells can serve as a new cellular source to regenerate new  $\beta$  cells following extreme  $\beta$  cell loss. Therefore, further understanding the mechanism underlying these findings may unlock new doors for  $\beta$  cell regeneration in diabetes therapies.

(1) Examine the adaption of  $\alpha$  cells in response to  $\beta$  cell injury. Previous studies and the results in this dissertation indicate that  $\alpha$  cells sense the injury of  $\beta$  cells, and facilitate  $\beta$  cell regeneration. The response of  $\alpha$  cells to  $\beta$  cell injury includes increased *glucagon* gene activity and transdifferentiated into  $\beta$  cells. However, it is still unclear how  $\alpha$  cells

sense the injury or loss of  $\beta$  cells. The following possibilities exist: direct cell-cell interaction,  $\beta$  cells release certain signals upon injury, imbalance of glucagon and insulin hormone ratio, indirect effects via altered metabolism upon lacking of insulin. Because the zebrafish pancreas resides in the endoderm region, it is challenging to perform *in vivo* imaging tracing to determine the dynamic interaction between  $\alpha$  and  $\beta$  cells under physiology condition as well as under  $\beta$  cell injury. Therefore, we can use *in vitro* endoderm culture to exploring this possibility. Using this approach, we can capture the complete process involved in  $\alpha$  to  $\beta$  cell transdifferentiation. Furthermore, we can combine genetic modification and pharmaceutical treatments to determine how cell-cell connections and extracellular signals influence  $\alpha$  cell response toward  $\beta$  cell injury. For example, the new regenerated pancreatic endocrine cells can form the long cellular membrane protrusions to connect with the existing islet cells (Chapter Three). Recent studies have shown that cellular protrusions not only help cell migration but also involved in long-distance cell signal transduction (422,423). Therefore, it will be highly interesting to test whether  $\alpha$  and  $\beta$  cells can communicate via dynamic cellular protrusions or whether this behaviors is required for islet regeneration. The *in vivo* time laps system developed here will thus be highly valuable. Besides the *in vitro* endoderm culture, the current advance of the light-sheet fluorescence microscopy may also make it possible to trace the behaviors of  $\alpha$  cells in response to injury in *in vivo* zebrafish models (424).

(2) Examine the genetic profile during  $\alpha$  to  $\beta$  cell transdifferentiation. In order to further explore the signaling pathways that directs  $\alpha$  to  $\beta$  cell transdifferentiation, large scale sequencing can be performed using the sorted  $\alpha$  and  $\beta$  cells in the non-regeneration or regeneration groups using *Tg(gcga:GFP); Tg(ins:dsRed)* transgenic fish lines. An alternative approach is to sort the *gcga:GFP<sup>+</sup>ins:dsRed<sup>+</sup>* cells in the regenerating islet, as these cells may represent the transdifferentiating stage of  $\alpha/\beta$  cells. We will then compare the phenotype of these transdifferentiating cells with the *gcga:GFP<sup>+</sup>* ( $\alpha$ ) cells and *ins:dsRed<sup>+</sup>* ( $\beta$ ) cells. Using this approach, we can define new genetic targets that are activated during the transdifferentiation process.

(3) Understanding the mechanisms by which pancreatic hormones maintain  $\alpha$  cell stability in mammalian systems. In order to verify whether, in mammalian systems,

pancreatic hormones play similar roles in governing  $\alpha$  cell fate, the mammalian  $\alpha$  cell line ( $\alpha$ TC) together with pharmaceutical approaches using glucagon and insulin signaling activator/inhibitors will be performed. Further genetic modification in these cell lines can be achieved either via siRNA or CRISPR techniques. Moreover, following these experiments, we will move these findings into *in vivo* rodent models. For example, we can test the effects of glucagon signaling on  $\alpha$  cell transdifferentiation using the *Tg(ins:DTR)* mice models which can induce extreme  $\beta$  cell ablation in the background of *Gcgr*<sup>-/-</sup> or *GLP-1r*<sup>-/-</sup>. Furthermore, this examination can be performed in human islets to test whether the plasticity of  $\alpha$  cells is present in humans, and whether glucagon/insulin signaling can manipulate this process.

(4) Understanding the downstream signaling mechanisms by which pancreatic hormones regulate  $\alpha$  cell fate. Our results demonstrate that insulin and glucagon signaling play important roles in directing the  $\alpha$  to  $\beta$  cell transforming process. It is expected that these signals may act on the innate genetic program to alter the expression of key  $\alpha$  and  $\beta$  transcription factors. Finding such a link between hormone signaling and transcription factor expression may be critical to provide a specific drug to promote  $\alpha$  cell transdifferentiation into  $\beta$  cells. Previous studies have shown that certain transcription factors, including Arx and Pdx1, can push this transformation process.. Therefore, these transcription factors may provide initial targets, and the genetic profiling approach detailed above will generate new transdifferentiation targets. We can then use an *in vivo* transgenic approach to activate specific signaling pathways in  $\alpha$  cells to verify that these processes play a role in  $\beta$  cell transdifferentiation. For example, we can over-express constitutional activated PKA in  $\alpha$  cells using the glucagon promoter to verify whether glucagon-induced PKA activation play an essential role in directing the  $\alpha$  cell fate switch. Furthermore, it is likely that insulin/*glucagon* hormones regulate  $\alpha$  cell fate changes via epigenetic machinery. Therefore, it will be interesting to analyze DNA methylation and histone modifications in the key transcription factors that are involved in  $\alpha$  and  $\beta$  cell fates, and examine whether alterations in the epigenetic profile in promote transdifferentiation.

## The disease models to achieve the future research directions

(1) Exploring the  $\beta$  cell regeneration mechanisms in partial  $\beta$  cell ablating models. Previous studies have shown that the mode of  $\beta$  cell regeneration is highly dependent upon injury models and the age of organisms. Previous studies in rodents indicate that  $\alpha$  cell transdifferentiation only occurs following extreme  $\beta$  cell ablation. Nevertheless, it is unknown whether in the zebrafish model, transdifferentiation of  $\alpha$  cells is also restricted to this extreme  $\beta$  cell ablation condition. Thus, it will be interesting to analyze whether  $\alpha$  cell transdifferentiation can occur under partial  $\beta$  cell ablation. We have determined that *Tg(gata5:loxp-mcherry-loxp-DTA); Tg(ins:cre)* can induce partial  $\beta$  cell ablation. Therefore, we will investigate the question above via *Tg(gata5:loxp-mcherry-loxp-DTA); Tg(ins:creER)* tamoxifen inducible  $\beta$  cell specific ablation system. If future studies reveal the restriction of  $\alpha$  cell transdifferentiation to extreme  $\beta$  cell ablation condition in zebrafish models, it would suggest that  $\beta$  cells are critical for maintaining  $\alpha$  cell fate stability. Other results in this dissertation indicate that insulin secreted from  $\beta$  cells may play such a role. Understanding the interaction between  $\alpha$  and  $\beta$  cells in order to maintain cell fate maintenance could path the way for a cure to diabetes.

(2)  $\beta$  cell regeneration under inducible inflammation models. This inflammation environment can be created by inducible pancreatic expression of immune factors such as IL-1 $\beta$  or IFN- $\gamma$ . Previous studies also show that immune cells, such as macrophages, may act as a double-edged sword in islet reorganizing and  $\beta$  cell regeneration. Thus, it is will interesting to determine the specific role of immune cells in regulating the sources of  $\beta$  cell. Zebrafish containing *hs:loxp-mcherry-loxp-IL-1 $\beta$ /IFN- $\gamma$ ; ins:cre* can be designed to selectively activate an inflammatory response in a time controlled manner. Further studies regarding immune cell infiltration and remodeling in the islet in response to the secreted immune factors can be pursued.

(3)  $\beta$  cell regeneration and compensation in adult zebrafish models.  $\beta$  cell regeneration in adult fish has been tested in previous published studies. Future  $\beta$  cell regeneration studies in adult fish can be carried on in STZ-induced  $\beta$  cell ablation model and MTZ-*Tg(ins:CFP-NTR)*  $\beta$  cell ablation models. Different from the embryonic fish, the adult fish pancreas is composed of secondary islets that arise from the ventral

pancreatic endocrine cells. Thus, it will be possible to dissect the potential different roles of dorsal and ventral derived  $\alpha$  cells in contributing to  $\beta$  cell regeneration. In addition to  $\beta$  cell specific ablation, we can also determine whether obesity or genetically induced insulin resistance can stimulate  $\beta$  cell compensation in adult fish. Moreover, previous studies have shown that the ability of  $\beta$  cells to regenerate decreases with age. Because of the fast life cycle of zebrafish, it will be interesting to analyze how aging influences pancreatic progenitor self-renewal and  $\beta$  cell regeneration in zebrafish models.

(4) Studies in mammalian diabetic systems. Although it is more expensive to perform experiments using mammalian systems such as rodent models, there are certain questions we cannot answer using a zebrafish model. For example, rodent models must be used to investigate the effects of insulin and glucagon on embryonic pancreas development during maternal gestational diabetes. Furthermore, it is also critical to verify whether the findings we observed in zebrafish models are conserved in mammalian systems as well. To do this, we can use rodent models that mimic human T1DM or T2DM to analyze the effects of pancreatic hormones on  $\beta$  cell development. Verifying such targets in rodent model will be a necessary step to move towards the cure for Diabetic patients. Besides using *in-vivo* rodent models, we can also perform *in-vitro* studies using human islet samples to test the roles of pancreatic hormones in islet endocrine cell fate specification. Furthermore, recent advances in human-stem cell research of *in-vitro*  $\beta$  cell differentiation also make it feasible to study the effects of the pancreatic hormones in regulating pancreatic progenitor cell differentiation. All together, through studies using diffenet model systems, it will bring better understanding for the roles of pancreatic hormone signaling pathways in regulating pancreas development and endocrine homeostasis, which will provide new therapeutic targets for  $\beta$  cell regeneration in Diabetic treatment.



APPENDICES

Appendix one: Zebrafish de-novo *dnmt3ab* is required for pancreas development

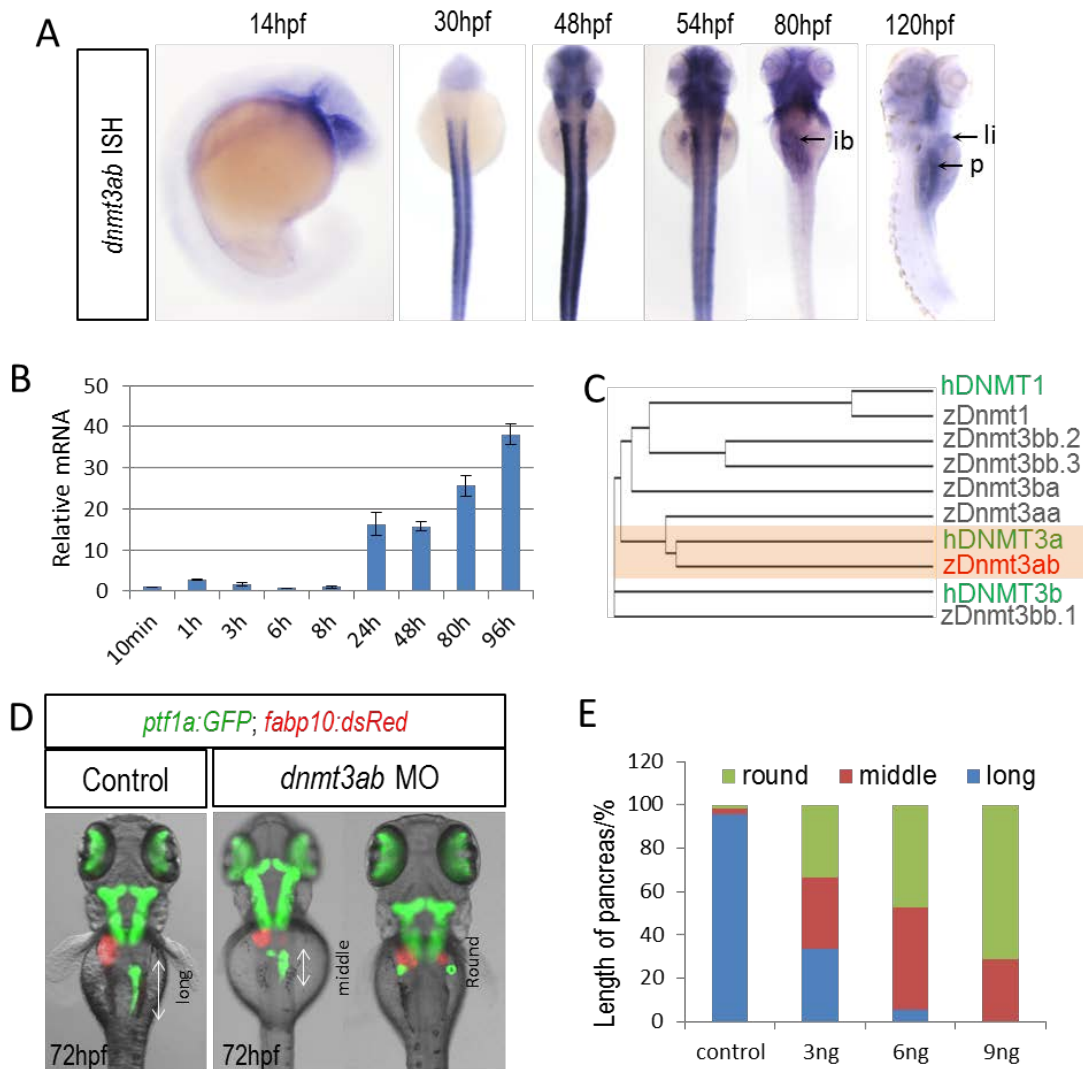


Figure 1. *dnmt3ab* knockdown induces small and duplicated pancreas.

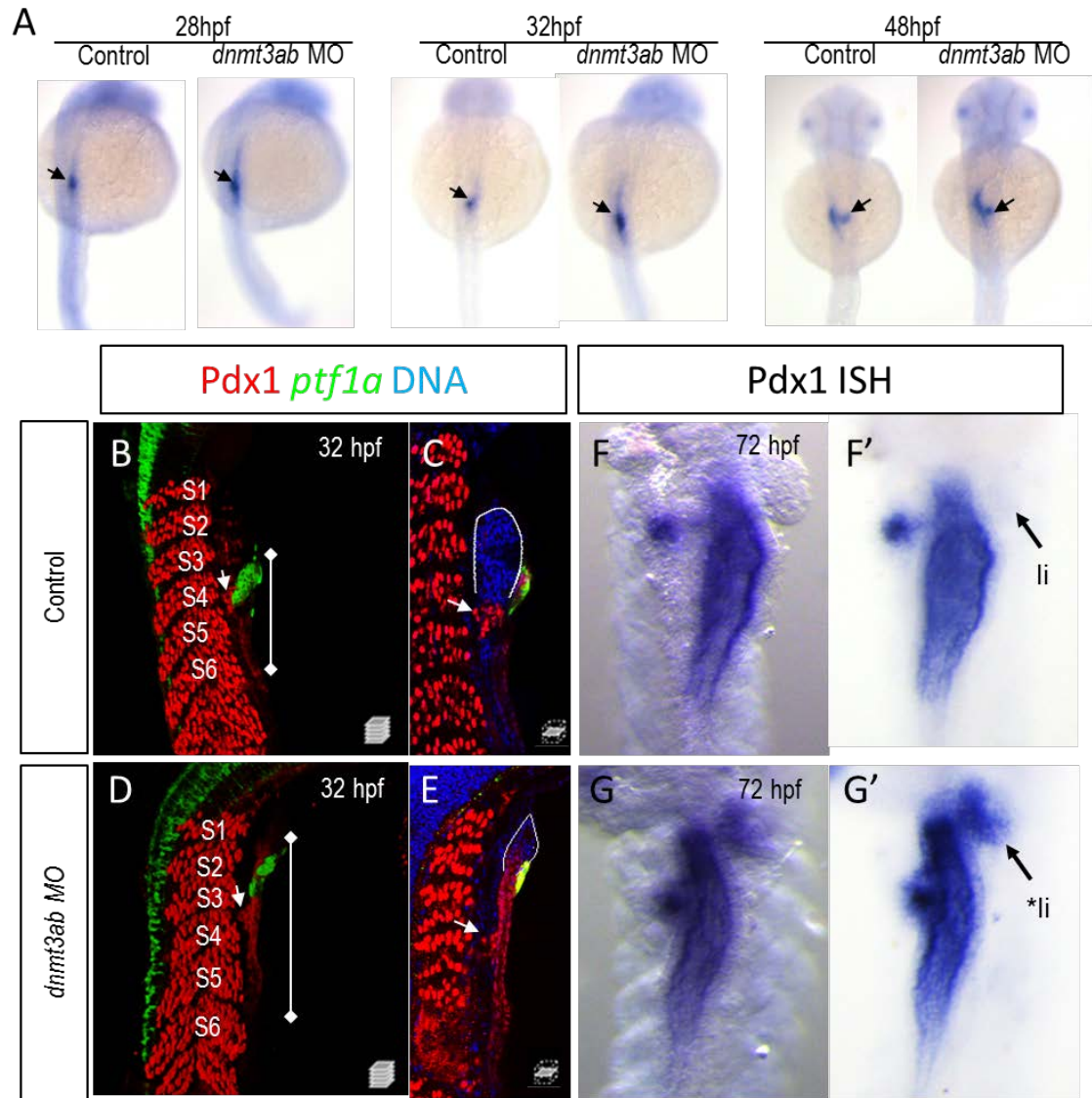


Figure 2. knockdown of *dnmt3ab* expand Pdx1 domain to anterior during development.

li: liver

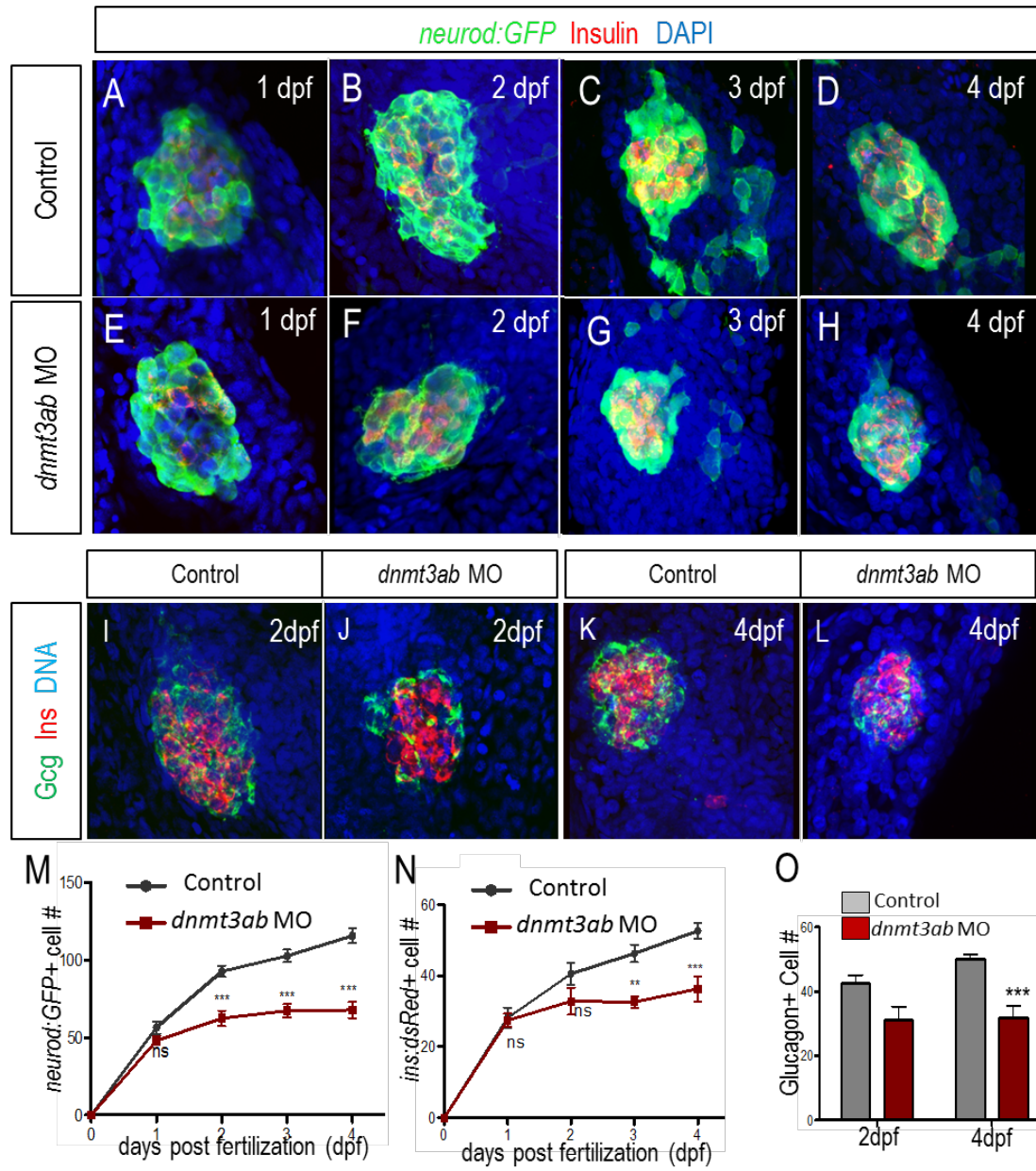


Figure 3. Knockdown of *dnmt3ab* decreases pancreatic endocrine cell number.

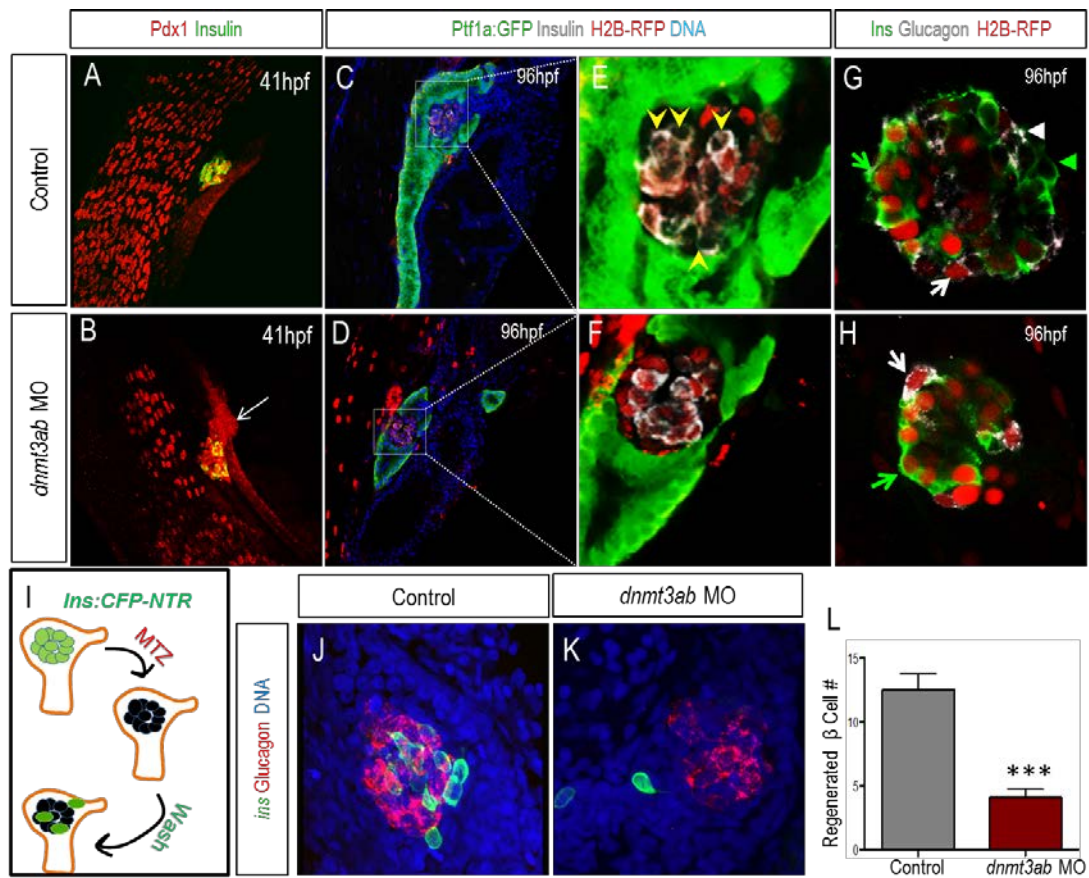


Figure 4. Knockdown of *dnmt3ab* blocks endocrine differentiation from ventral pancreas.

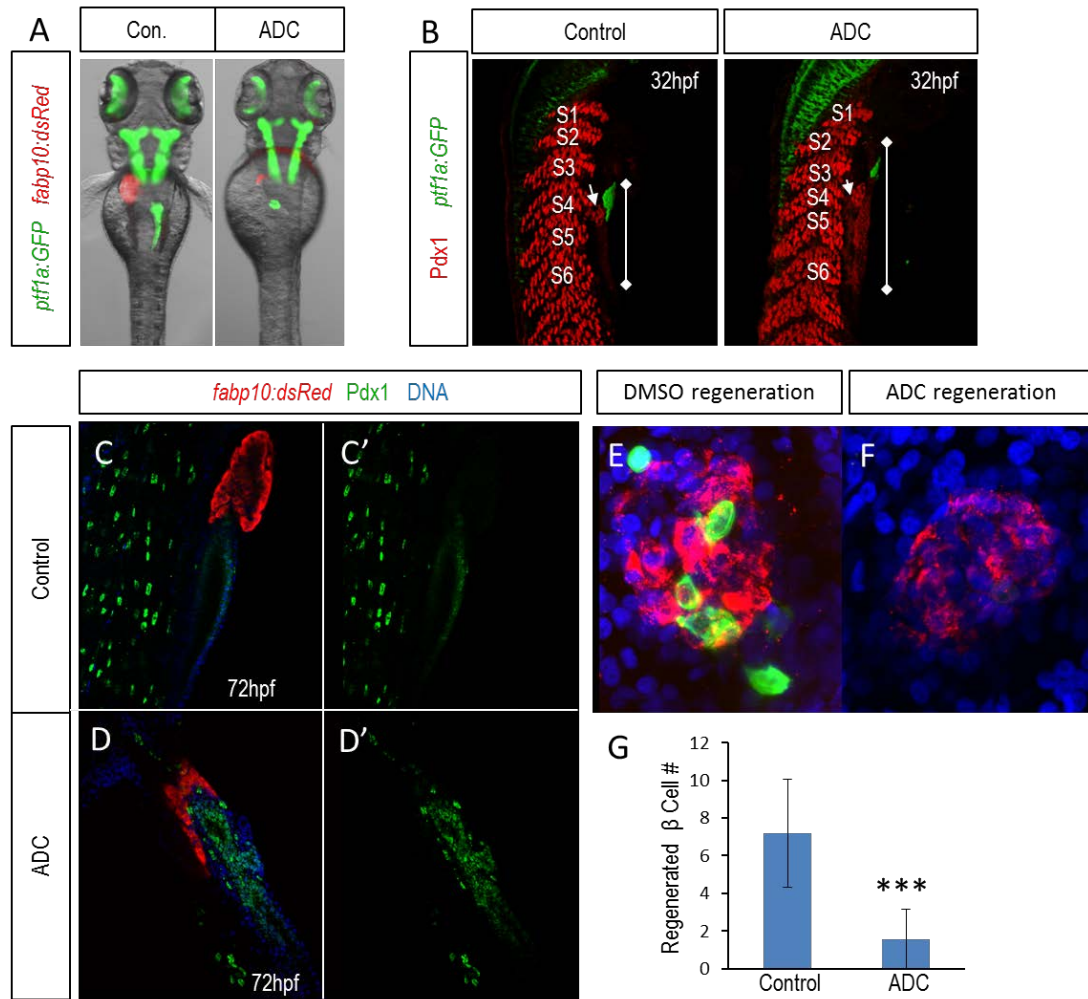


Figure 5. Treatment of Dnmts inhibitor ADC mimics *dnmt3ab* morphants phenotype.



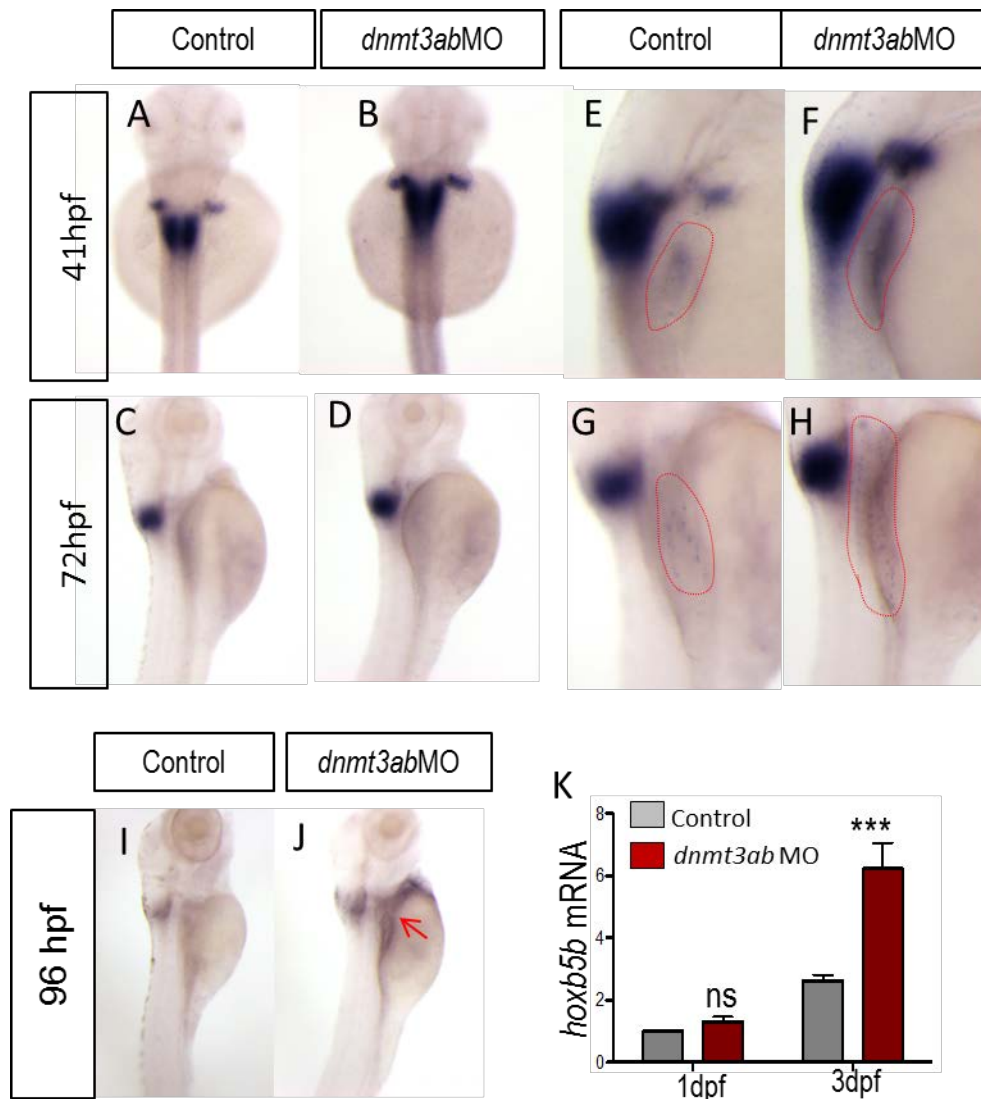


Figure 6. knockdown of *dnmt3ab* increase endoderm expression of retinoic acid effector *hoxb5b* during development.

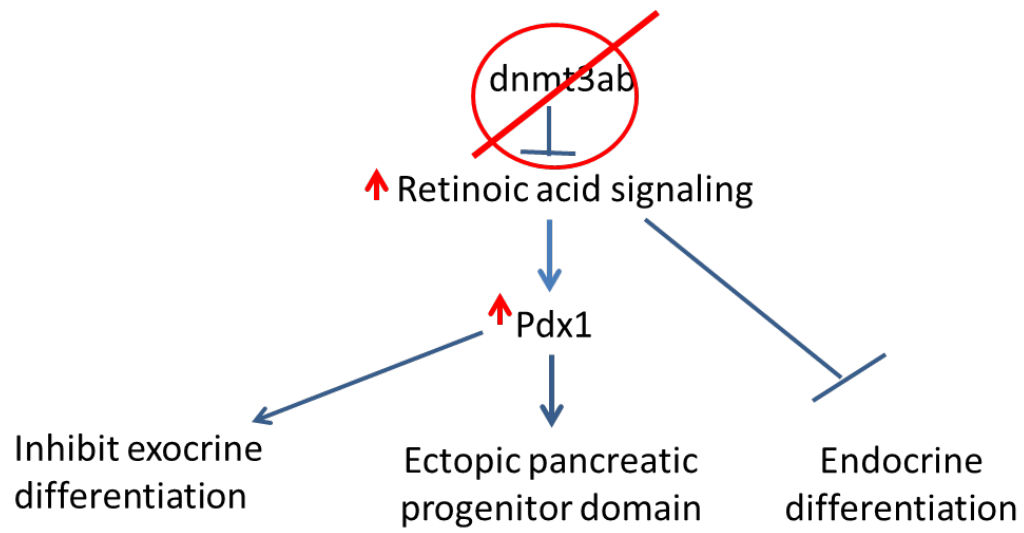


Figure 7. Model figure for dnmt3ab regulated pancreas development via retinoic acid signaling.

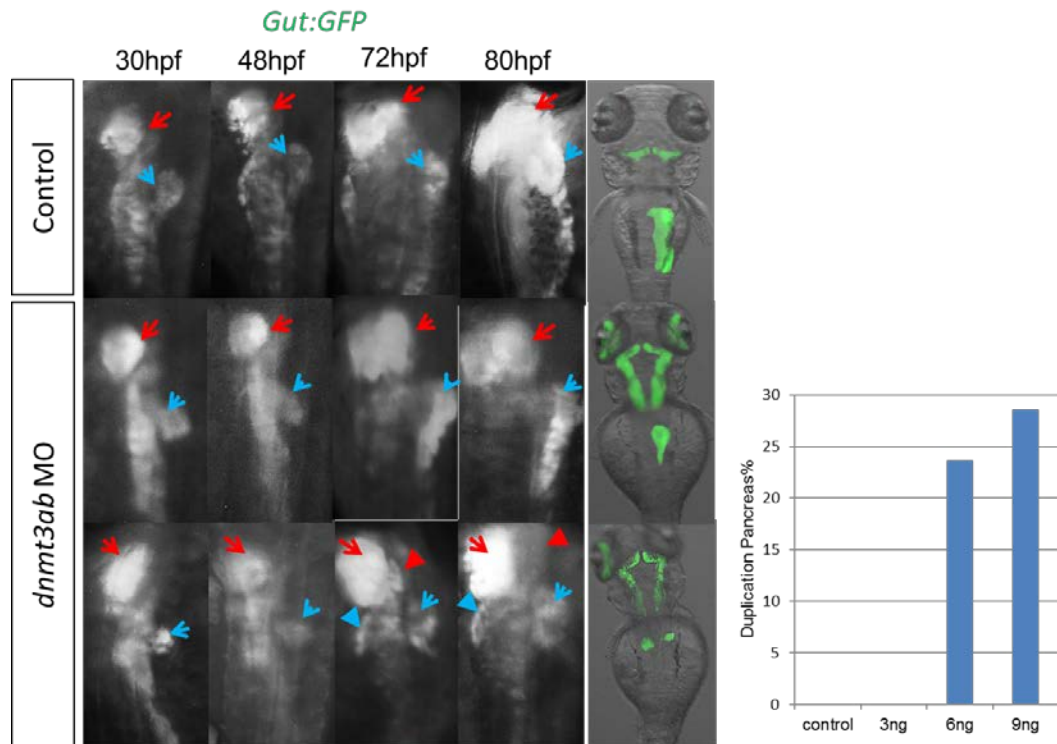


Figure 8. Knockdown of *dnmt3ab* induce formation of duplicated pancreas.



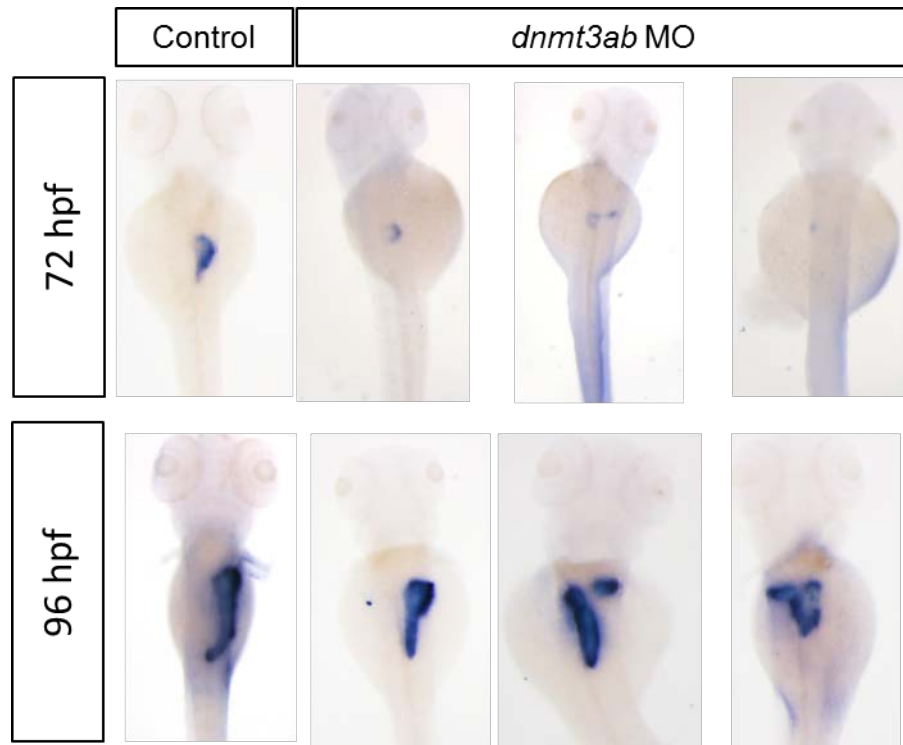


Figure 9. Knockdown of *dnmt3ab* reduce trypsin expression in pancreas.

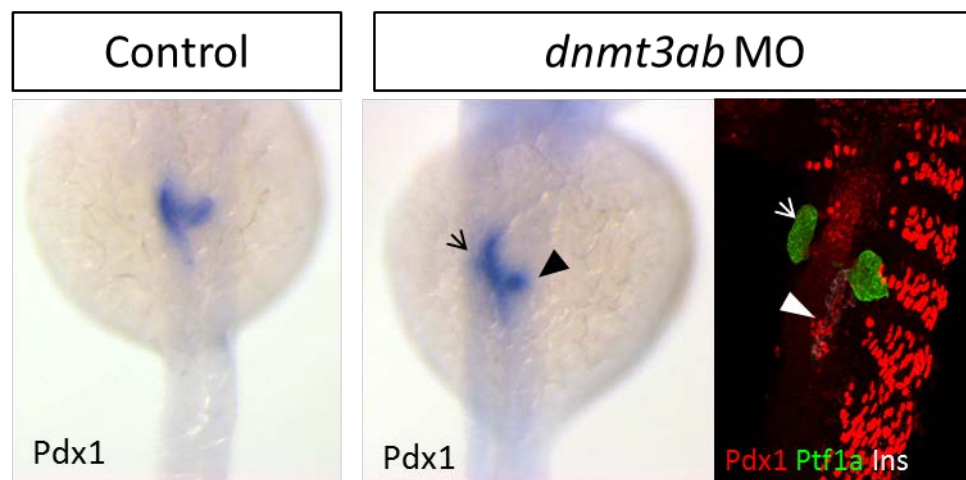


Figure 10. Knockdown of *dnmt3ab* induce extra-pancreas bud from Pdx1+ endoderm domain

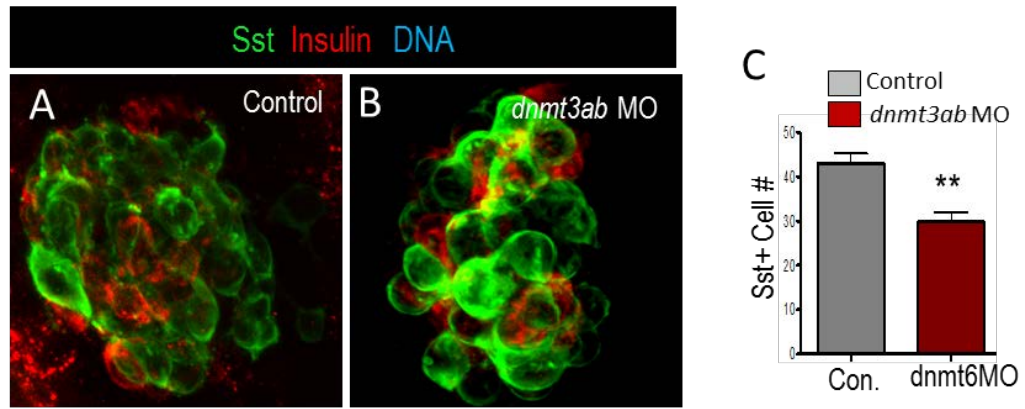


Figure 11. Knockdown of *dnmt3ab* reduces  $\delta$  cell number.

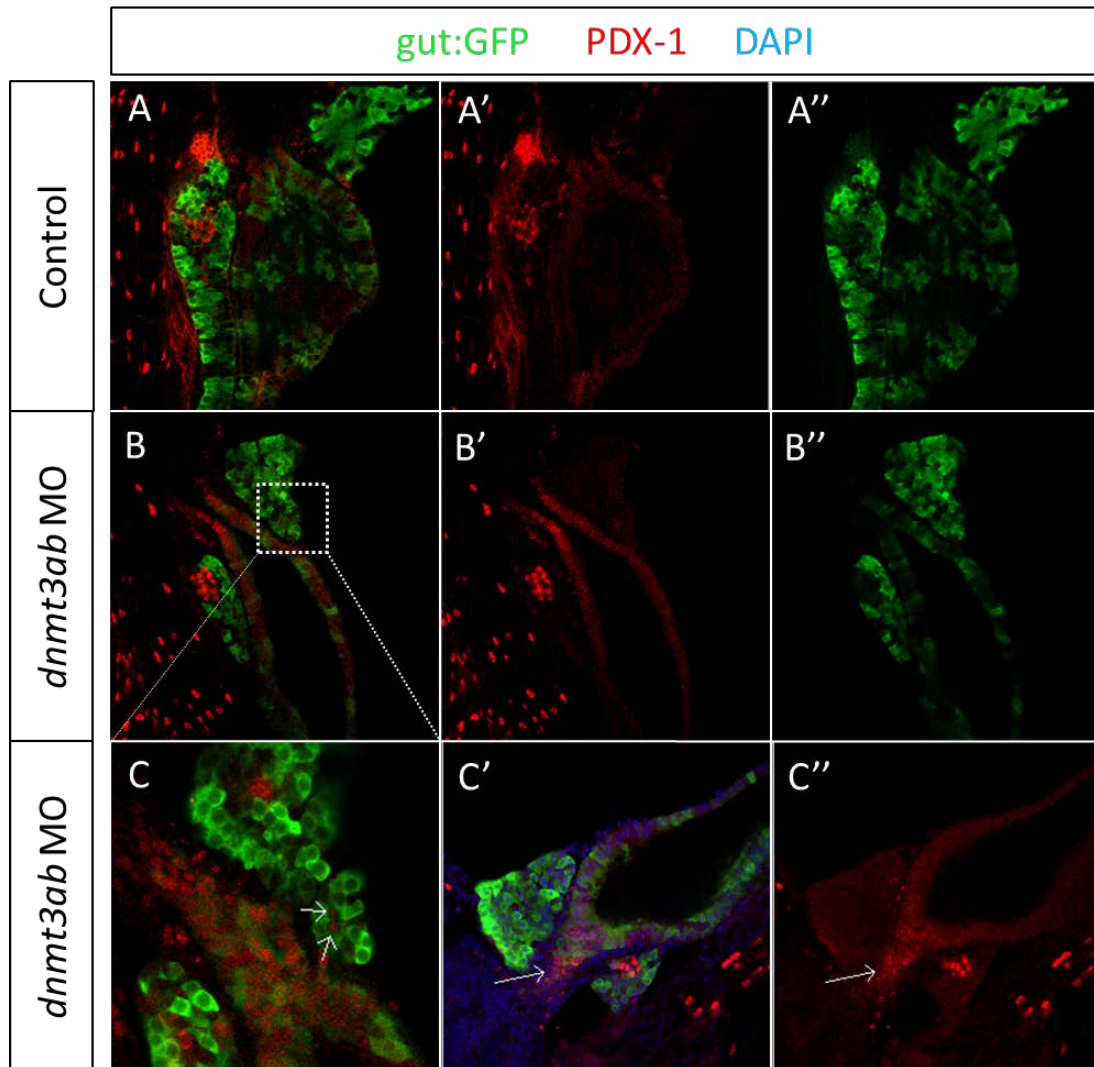


Figure 12. Knockdown of *dnmt3ab* increases Pdx1 expression in ventral endoderm and liver domain.

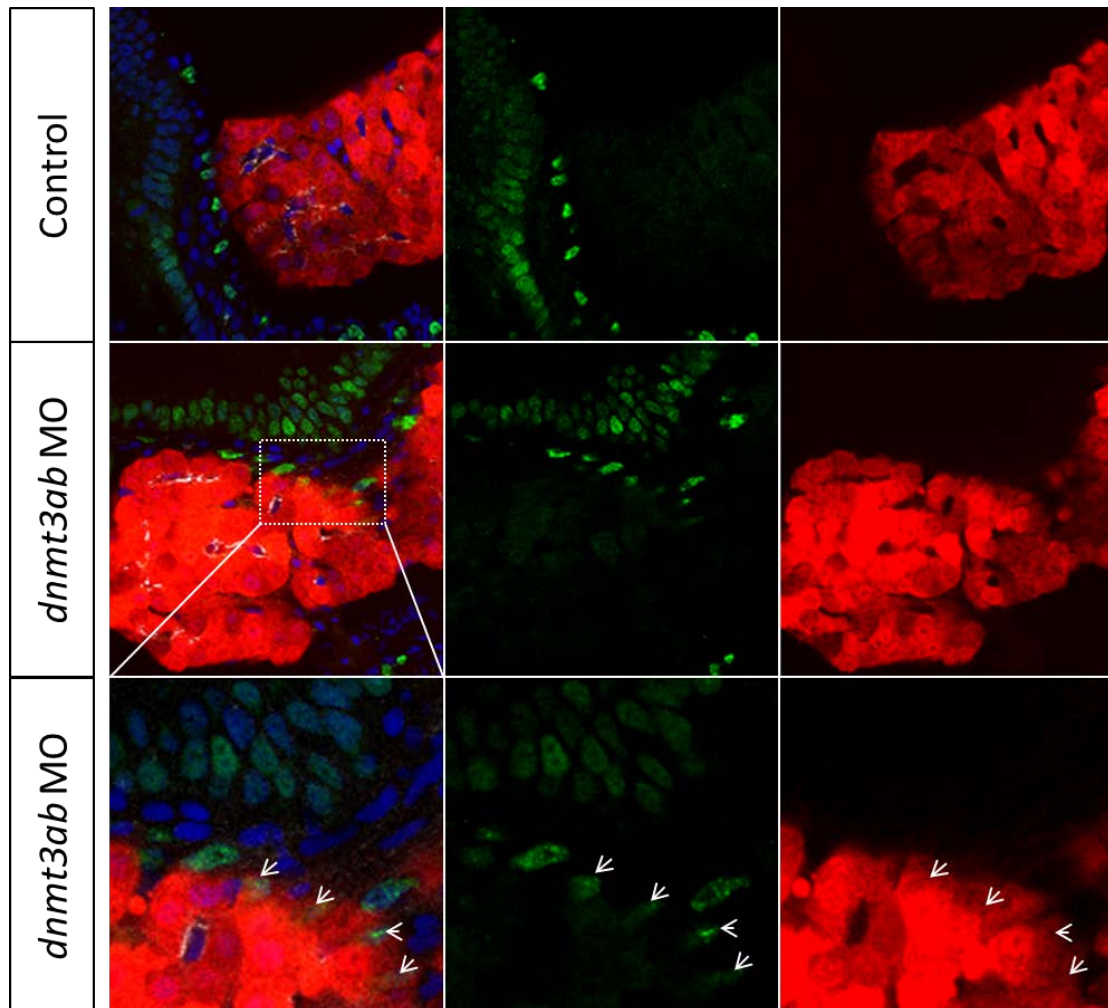


Figure 13. Knockdown of *dnmt3ab* induces ectopic Pdx1 expression in liver cells.

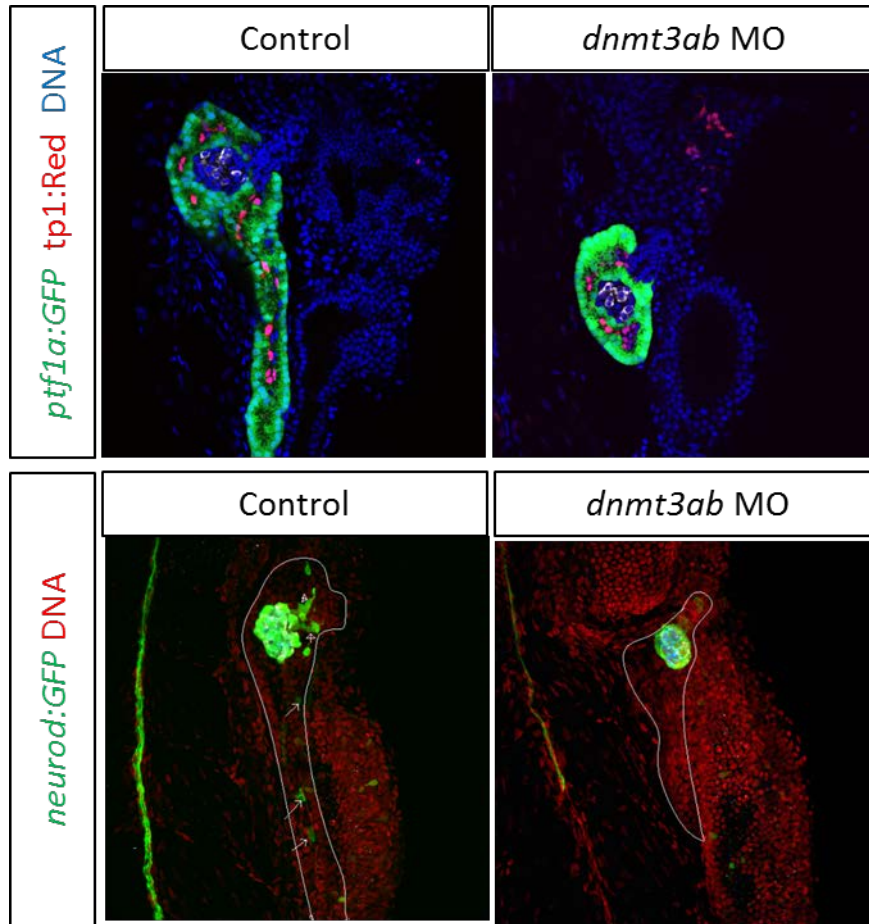


Figure 14. Reduced pancreatic duct and endocrine progenitor formation from *dnmt3ab* knockdown.

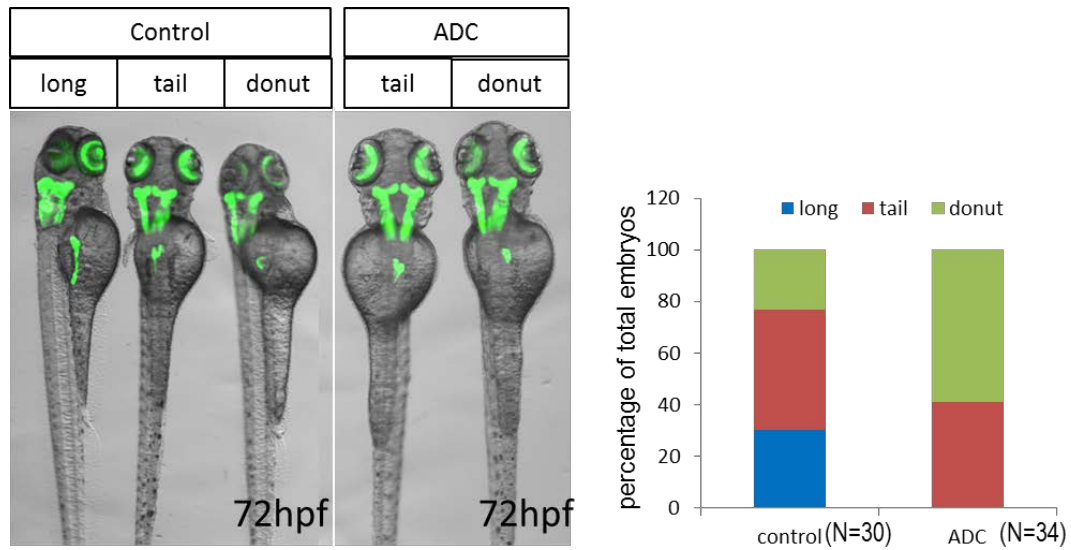


Figure 15. Treatment of Dnmts inhibitor ADC reduces pancreas size

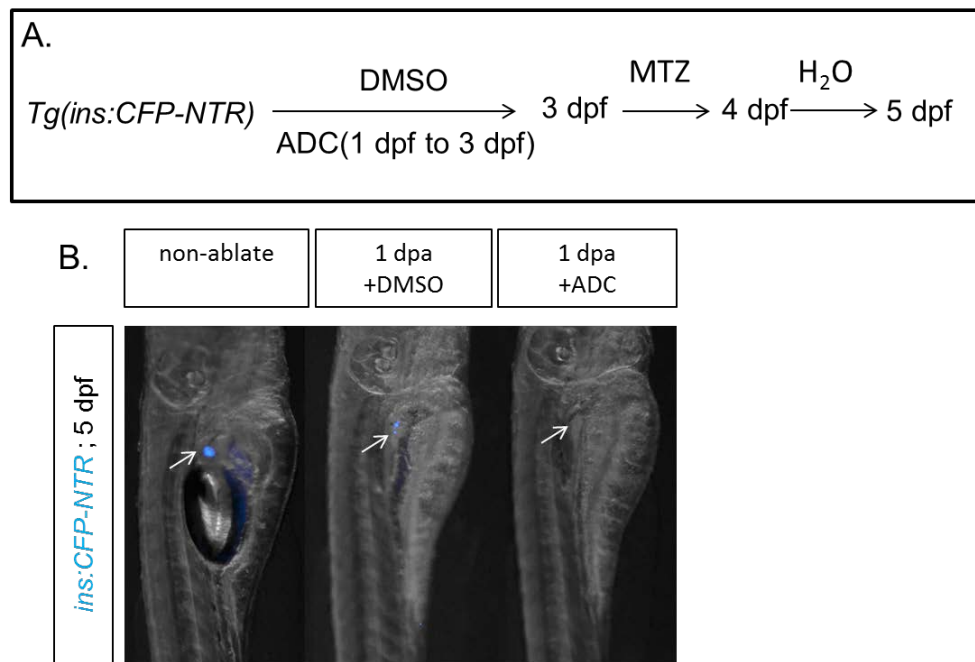


Figure 16. Pre-treatment of Dnmts inhibitor ADC impairs  $\beta$  cell regeneration

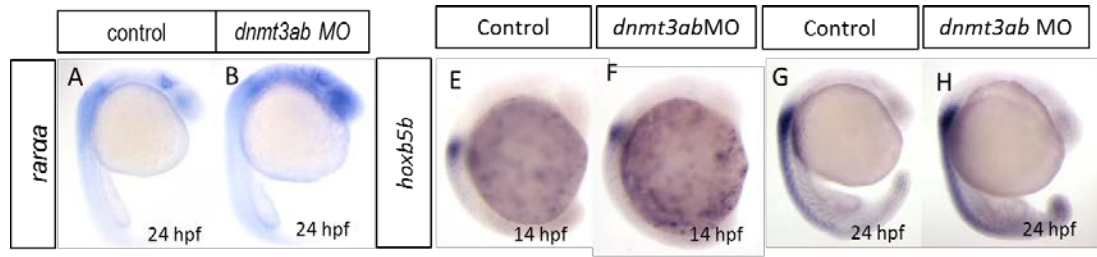


Figure 17. Knockdown of *dnmt3ab* altered retinoic signaling effector expression.



**Appendix two: The role of maintenance dnmt1 is regulating  $\alpha$  cell fate stability and  $\beta$  cell regeneration**

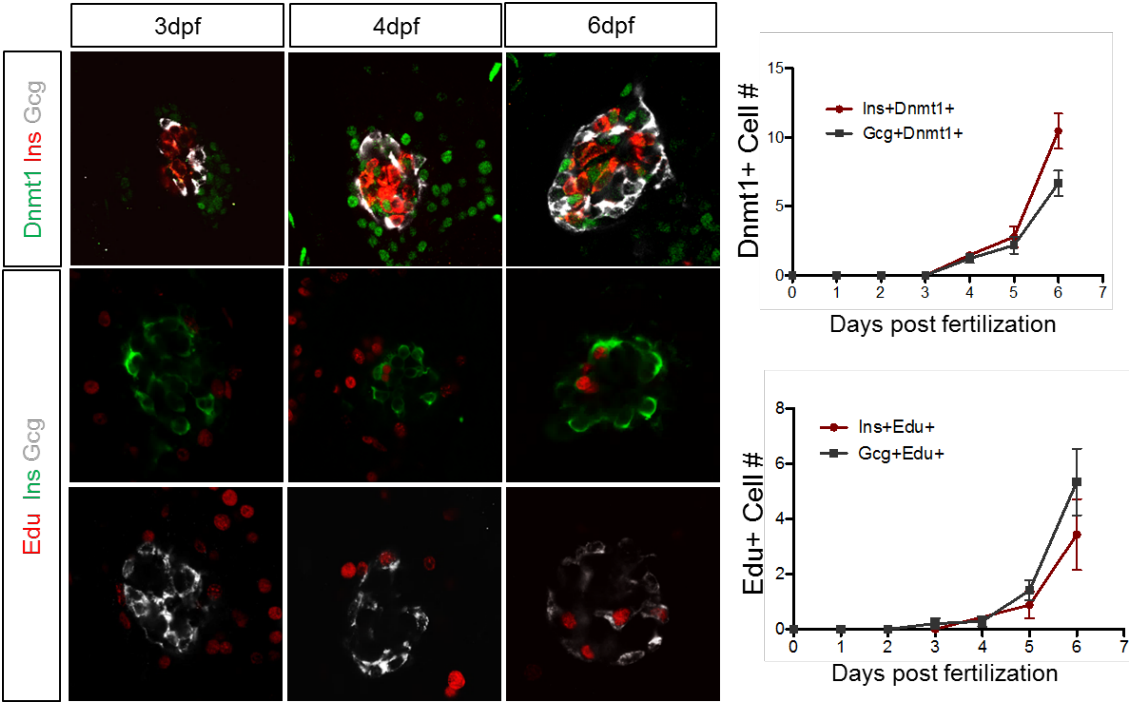


Figure1. Expression of Dnmt1 in  $\alpha$  and  $\beta$  cells is co-related with endocrine cell proliferation.



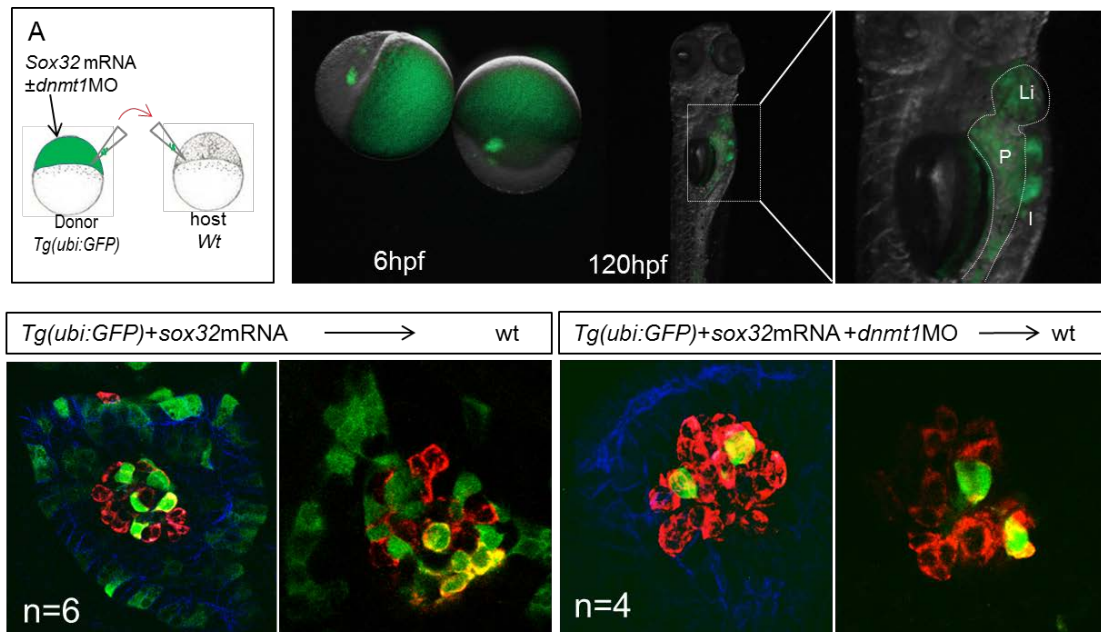


Figure2. *dnmt1* depleting cells differentiated into pancreatic endocrine cells but not exocrine cells.

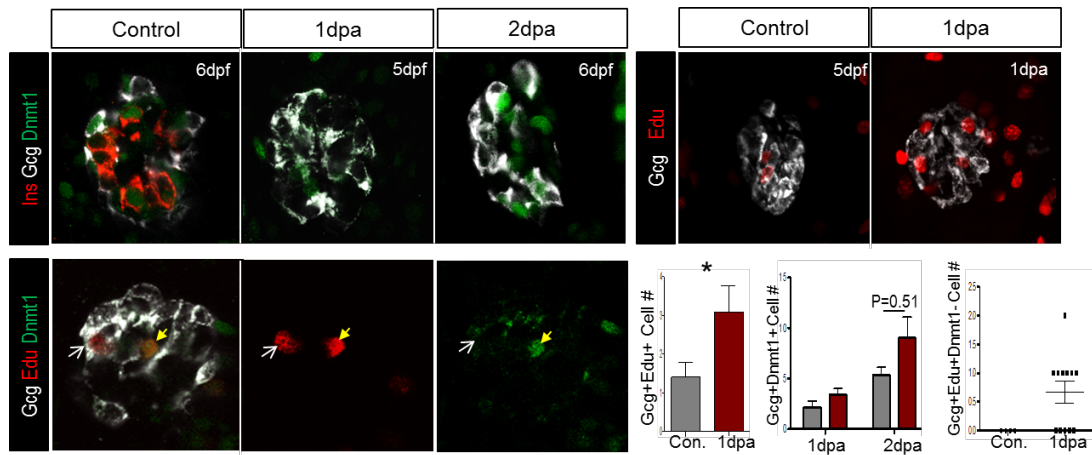


Figure3.  $\alpha$  cells increased proliferation but not Dnmt1 expression at 1 dpa  $\beta$  cell regenerating phase.

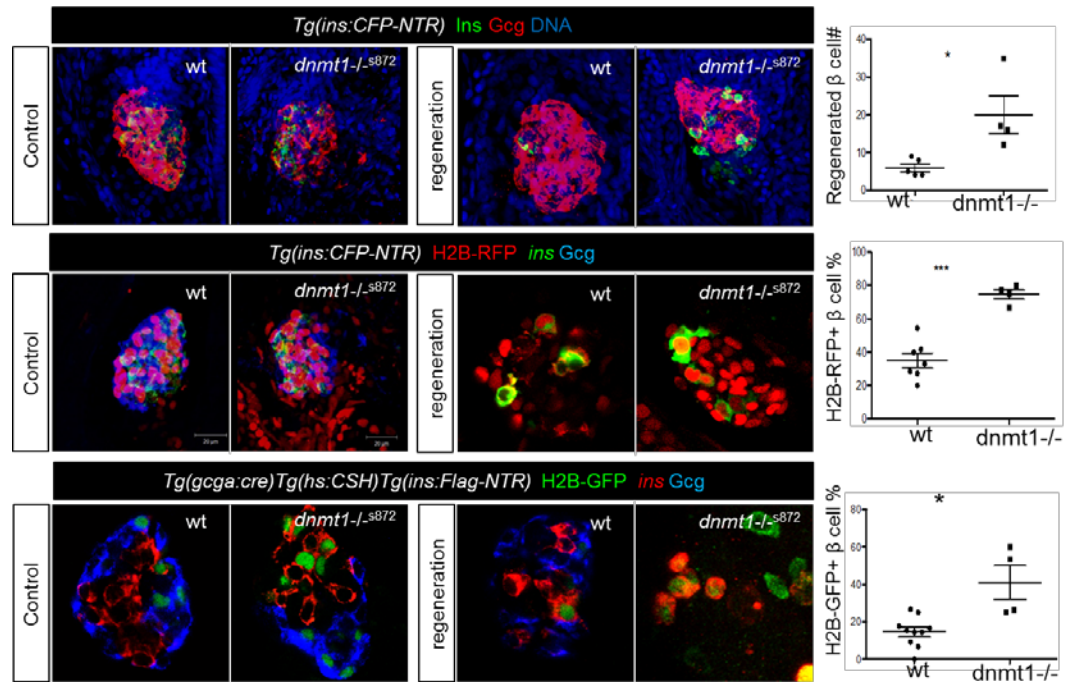


Figure4. Loss of *dnmt1* increases  $\alpha$  cell transdifferentiate into  $\beta$  cells following  $\beta$  cell ablation.

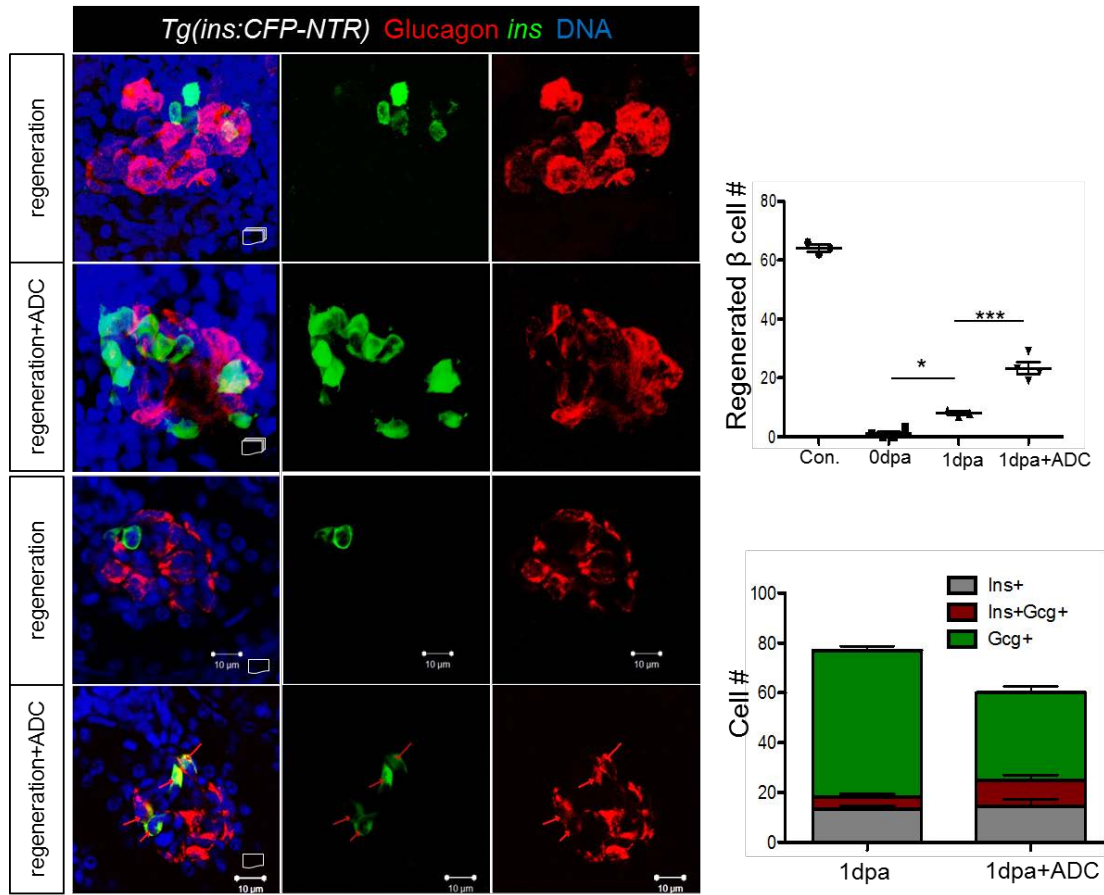


Figure5. Treatment of Dnmt1 inhibitor ADC increases  $\beta$  cell regeneration.

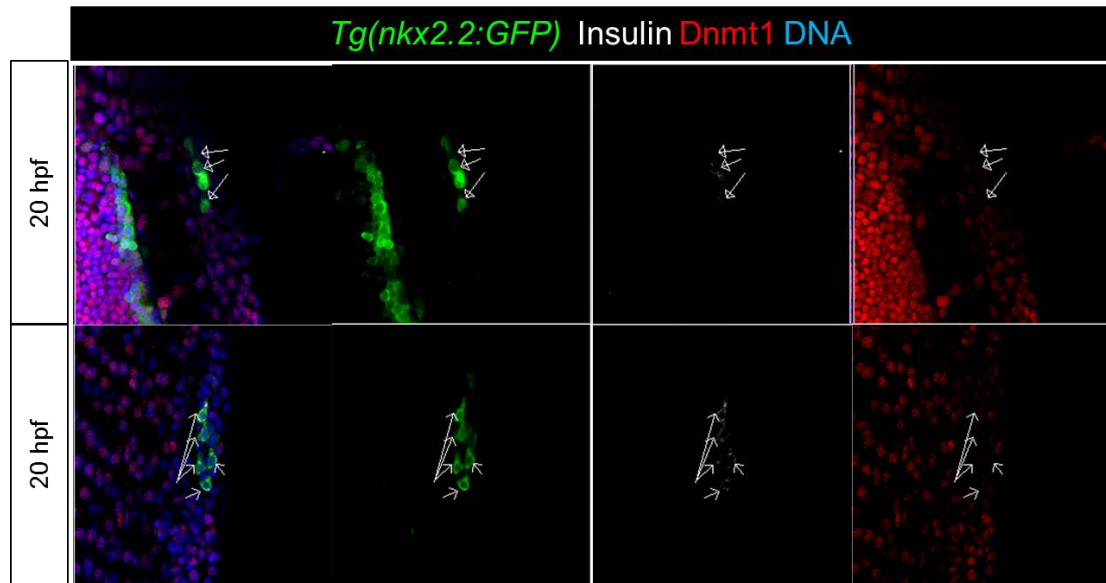


Figure 6. Dnmt1 is not expressed in the dorsal pancreas derived  $\beta$  cells.

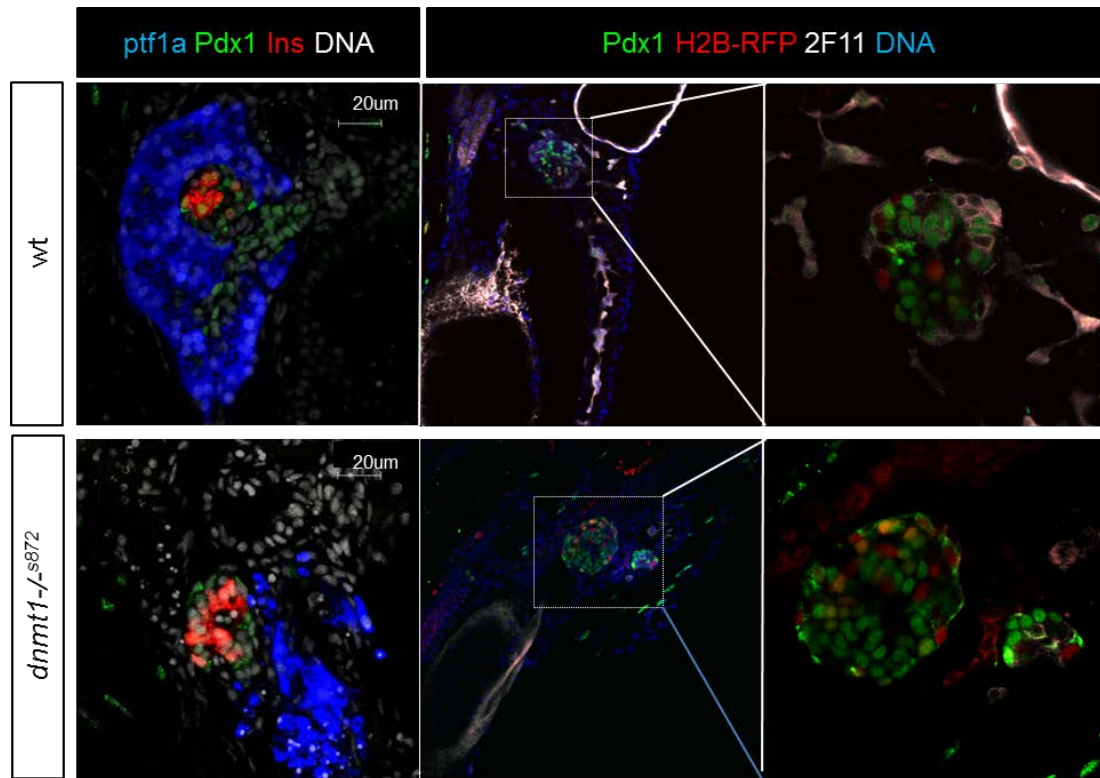


Figure 7. Remaining of H2B-RFP+ and H2B-RFP- Pdx1 expression in *dnmt1*<sup>-/-</sup> principal islet and extra-pancreatic duct.

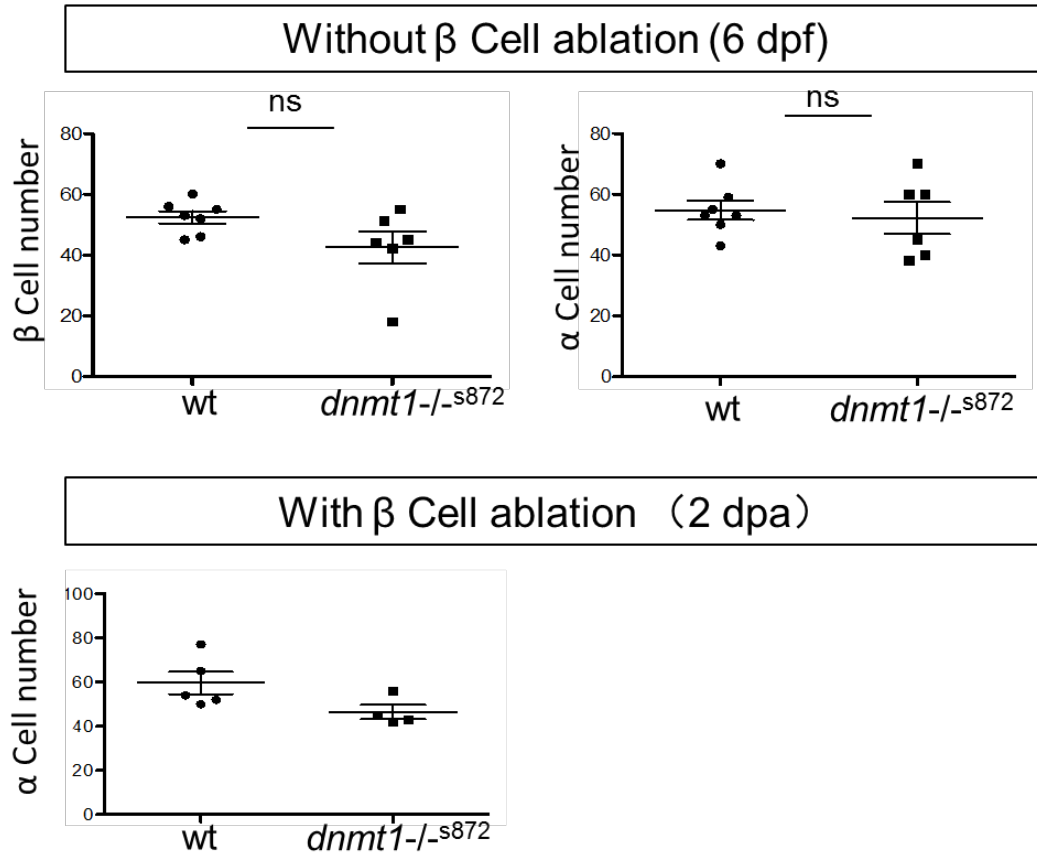


Figure 8. *dnmt1*<sup>-/-</sup> does not influence endocrine cell number during development.

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422. Wakayama, Y., Fukuhara, S., Ando, K., Matsuda, M., and Mochizuki, N. (2015) Cdc42 mediates Bmp-induced sprouting angiogenesis through Fmnl3-driven assembly of endothelial filopodia in zebrafish. *Developmental cell* **32**, 109-122
423. Phng, L. K., Stanchi, F., and Gerhardt, H. (2013) Filopodia are dispensable for endothelial tip cell guidance. *Development* **140**, 4031-4040
424. Ahrens, M. B., Orger, M. B., Robson, D. N., Li, J. M., and Keller, P. J. (2013) Whole-brain functional imaging at cellular resolution using light-sheet microscopy. *Nature methods* **10**, 413-420

## CURRICULUM VITAE

**Lihua Ye**

### **Education**

- 2010-2015      **Doctor of Philosophy**, Cellular and Integrative Physiology  
Indiana University, Indianapolis, IN
- 2008-2010      **Master of Science**, Chemical Biology  
Peking University, Beijing, China
- 2004-2008      **Bachelor of Science**, Pharmaceutical Sciences  
Peking University, Beijing, China

### **Research Experience**

- 2010-2015      **Indiana University School of Medicine, Indianapolis**  
Ph.D. in Dr. Ryan Anderson's Laboratory  
- Investigated insulin and glucagon hormones in regulating pancreatic  $\beta$  cell formation and regeneration using zebrafish models.
- 2008-2010      **Peking University, Beijing**  
Graduate student in Dr. Kui Wang's Laboratory  
-Investigated the toxic effects of Gadolinium compounds on human hepatocytes.

## **Publications**

1. *glucagon* is essential for  $\alpha$  cell transdifferentiation and  $\beta$  cell neogenesis.

**Ye L**, Robertson MA, Hesselton D, Stainier DY, Anderson RA\*,  
*Development*, 2015, 142:1407-1417, Highlighted as a feature article.

2. Insulin signaling regulates pancreatic progenitor cell differentiation.

**Ye L**, Robertson MA, Teresa LM, Anderson RA\*,  
*Development Biology*, 2015, in review.

3. Insulin signaling is required for the exocrine pancreas formation and acinar cell fate maintenance.

**Ye L**, Robertson MA, Teresa LM, Anderson RA\*, In preparation

4. Hepatocyte Growth Factor signaling in intra pancreatic ductal cells drives pancreatic morphogenesis.

Anderson RM, Delous M, Bosch JA, **Ye L**, Robertson MA, Hesselton D, Stainier DY\*  
*Plos Genetics* 2013, 9(7): e1003650.

5. GdCl<sub>3</sub> induced HepG2 cells apoptosis through Mitochondrial and External Death Pathways without Significant Elevation of ROS Generation.

**Ye L**, Zhe S, Yang X, Liu H, Wang K\*,  
*Biological Trace Element Research*, 2013, 151(1): 148-155

6. Gadolinium induced apoptosis of human embryo liver L02 cell line by ROS-mediated AIF pathway.

**Ye L**, Zhe S, Yang X, Liu H, Wang K\*,  
*Journey of Rare Earths*, 2011, 29(2): 178-184

7. Vanadium compounds induced mitochondria permeability transition pore (PTP) opening related to oxidative stress.

Zhao Y, **Ye L**, Zhe S, Wang K, Liu H, Yang X, Wang K\*,  
*Journal of Inorganic Biochemistry*, 2010, 04(4): 317-318

8. Quercetin and green tea polyphenols inhibit the mitochondrial damages and cytotoxicity induced by VO(acac)<sub>2</sub>.

Zhao Y, Zhe S, **Ye L**, Wang K, Liu H, Yang X, Wang K\*,  
*Journal of Chinese Pharmaceutical Sciences* 2009, 8 (3): 221-225

### **Abstracts and Presentation**

1. **Ye L**, Robertson MA, Anderson RA. (2015) Insulin signaling regulates pancreatic progenitor cell differentiation. 5<sup>th</sup> Annual Meeting of the Indiana Physiology Society (talk).

2. **Ye L**, Robertson MA, Hesselton D, Stainier DY, Anderson RA. (2014) Glucagon signaling is essential for  $\beta$  cell neogenesis and transdifferentiation from  $\alpha$  cells in zebrafish. 73<sup>th</sup> Annual Society for Developmental biology Meeting (poster).

3. **Ye L**, Robertson MA, Hesselton D, Stainier DY, Anderson RA. (2014) Glucagon signaling is essential for  $\beta$  cell neogenesis and transdifferentiation from  $\alpha$  cells in zebrafish. 52<sup>th</sup> Annual Midwest Society for development biology meeting (talk).

4. **Ye L**, Robertson MA, Anderson RA. (2014) Insulin signaling directs pancreas development via Pdx1 regulation. 5<sup>th</sup> Annual Midwest Graduate Research Symposium (talk).

5. **Ye L**, Robertson MA, Anderson RA. (2013) Glucagon is critical for  $\beta$  cell regeneration in zebrafish model. 6<sup>th</sup> Annual Midwest islet club conference (talk).
6. **Ye L**, Anderson RA. (2012) Inhibition of DNA methylation promotes  $\beta$  cell regeneration in zebrafish model. Inhibition of DNA methylation promotes  $\beta$  cell regeneration in zebrafish model. 50<sup>th</sup> Annual Midwest Society for development biology meeting (poster).
7. **Ye L**, Robertson MA, Anderson RA. (2012) Inhibition of DNA methylation promotes  $\beta$  cell regeneration in zebrafish model. 10<sup>th</sup> international zebrafish conference (poster).
8. **Ye L**, Robertson MA, Anderson RA. (2014) Glucagon is critical for  $\beta$  cell regeneration in zebrafish model. 1<sup>st</sup> KOI zebrafish meeting (talk).
9. **Ye L**, Robertson MA, Anderson RA. (2014) Insulin signaling directs early pancreatic progenitor differentiation. IUPUI Research Day (poster).
10. **Ye L**, Robertson MA, Anderson RA. (2013) The endocrine control of pancreatic  $\beta$  cell regeneration. IUSM Center for Diabetes Research Seminar Series (invited talk).
11. Gao Z, Zhao Y, **Ye L**, Yang X, Wang K. (2009) Interaction of Vanadium Compounds with Mitochondria. *J Biol Inorg Chem*, 14(suppl 1):S21-S27.
12. **Ye L**, Zhe S, Yang X, Liu H, Wang K. (2009) GdCl<sub>3</sub> induce the apoptosis of HepG2 cells via mitochondrial pathway, 10<sup>th</sup> Chinese National Conference on Bioinorganic Chemistry (poster).

13. Gao Z, Zhao Y, **Ye L**, Yang X. (2009) Hypoglycemic vanadium compounds: cellular protection versus toxicity, 14<sup>th</sup> International Conference on Biological Inorganic Chemistry (poster).

### **Honors and Awards**

2014-2015	T32 Diabetes and Obesity Training Fellowship
2014	IUPUI Graduate Professional Education Grant
2014	73 <sup>th</sup> Annual SDB Travel grant
2013	Aquaneering Arts of Science Photomicrography Contest, Third Prize
2010-2011	Indian University Graduate Student Fellowship
2008	Peking University First grade scholarship for excellent medical student
2007	Peking University Wearnes medical education scholarship
2006	Peking University General electric medical education scholarship
2005	Peking University Second grade scholarship for excellent medical student

### **Teaching**

2014	Indiana University School of Medicine, F503 Human Physiology Lecture: "Growth hormone and thyroid hormone" and "The endocrine pancreas"
2014	Indiana University Purdue University Indianapolis, BIOL55900 Endocrinology Lecture: "GI hormone" and "Pancreatic hormone"
2014	Indiana University Purdue University Indianapolis, BIOL697 Epeigenetics

Lecture: “mammalian DNA methylation” and “DNA demethylation”

2014-2015 Indianapolis Lighthouse Collage Preparatory Academy Program,  
Activity: zebrafish  $\beta$  cell regenerating demonstrating, diabetes lecturer

2014-2015 Molecular Medicine in Action Program,  
Lecture: “Using mouse & zebrafish models to find therapies for Diabetes”

2011-2015 Molecular Medicine in Action Program, Team member  
Activity: lab tour, experiment demonstration

2008 Peking University School of Pharmaceutical Science  
Lab instructor: General Chemistry

### **Service and Professional Memberships**

2015 Science judge for 27<sup>th</sup> Annual Hoosier Science and Engineering Fair

2014-2015 Indiana University Gateway Ph.D. Program, student mentor

2012-2015 member of Society for Development Biology

2013-2015 member of American Heart Association